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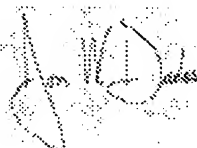
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. **EE068741456US****INVENTOR(S)**

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☒ Additional inventors are being named on the _____ separately numbered sheets attached hereto**TITLE OF THE INVENTION (500 characters max)****METAL COMPLEXES OF N-HETEROCYCLIC CARBENES AS
RADIOPHARMACEUTICALS AND ANTIBIOTICS**

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330-376-4577**ENCLOSED APPLICATION PARTS (check all that apply)**☒ Specification Number of Pages**69**☐ CD(s), Number☐ Drawing(s) Number of Sheets☐ Application Data Sheet. See 37 CFR 1.76☒ Other (specify)**postcard****METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT**☒ Applicant claims small entity status. See 37 CFR 1.27.☐ A check or money order is enclosed to cover the filing fees☒ The Commissioner is hereby authorized to charge filing
fees or credit any overpayment to Deposit Account Number:**50-0959**☐ Payment by credit card. Form PTO-2038 is attached.**FILING FEE
AMOUNT (\$)****\$80.00**The invention was made by an agency of the United States Government or under a contract with an agency of the
United States Government.☒ No.☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

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(if appropriate)**26,615**TELEPHONE **330-376-2700**

Docket Number:

089498-0463**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number

089498-0463

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Number 2 of 2

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of

WILEY J. YOUNGS et al.

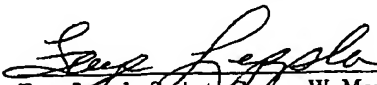
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For METAL COMPLEXES OF N-
HETEROCYCLIC CARBENES
AS RADIOPHARMACEUTICALS
AND ANTIBIOTICS

CERTIFICATE OF MAILING
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TRANSMITTAL SHEET

Enclosed are the following documents:

Provisional Application Cover Sheet

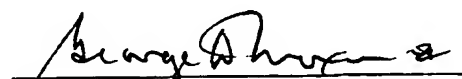
Provisional Patent Application

Return Receipt Postcard

AUTHORIZATION TO CHARGE DEPOSIT ACCOUNT

The Director is hereby authorized to charge payment of any fees associated with this communication or credit any overpayment to Deposit Account No. 50-0959 (089498-0463).

Respectfully submitted


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September 5, 2003

METAL COMPLEXES OF N-HETEROCYCLIC CARBENES AS
RADIOPHARMACEUTICALS AND ANTIBIOTICS

**STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT**

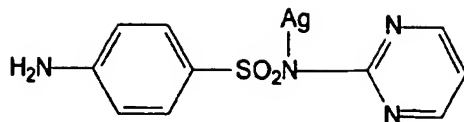
This invention was made with government support under a grant, Award Number NIH R15 CA 96739-01. The government may have certain rights to the invention.

BACKGROUND OF THE INVENTION

This invention relates to metal-containing, therapeutic, antimicrobial, and antifungic compounds. More particularly, this invention relates to metal complexes of N-heterocyclic carbenes and their use as antimicrobial agents, antifungic agents and radiopharmaceutical compositions.

Silver has long been used for its antimicrobial properties. This usage predates the scientific or medical understanding of its mechanism. For example, the ancient Greeks and Romans used silver coins to maintain the purity of water. Today silver is still used for this same purpose by NASA on its space shuttles. Treatment of a variety of medical conditions using silver nitrate was implemented before 1800. A 1% silver nitrate solution is still widely used today after delivery in infants to prevent gonorrheal ophthalmia. Since at least the later part of the nineteenth century, silver has been applied in a variety of different forms to treat and prevent numerous types of bacteria related afflictions.

Other treatments, such as the application of silver foil to post surgical wounds to prevent infection survived as a medical practice into the 1980's in Europe, and silver nitrate is still used as a topical antimicrobial agent. In the 1960's the very successful burn treatment silver complex, silver sulfadiazine, shown in formula 1 below, was developed. Commercially known as Silvadene® Cream 1%, this complex has remained one of the most effective treatments for preventing infection of second and third degree burns. Silver sulfadiazine has been shown to have good antimicrobial properties against a number of gram-positive and gram-negative bacteria. It is believed that the slow release of silver at the area of the superficial wound is responsible for the process of healing. Studies on surgically wounded rats have shown the effectiveness of both silver nitrate and silver sulfadiazine to aid in the healing process. By using these common silver antimicrobial agents, inflammation and granulation of wounds were reduced, although the complete mechanism for these phenomena is not understood.



1 Silver Sulfadiazine.

Recently developed silver-coating techniques have lead to the creation of a burn wound dressing called Acticoat. The purpose of this dressing is to avoid adhesion to wounds while providing a barrier against infection. Some clinical trials have also demonstrated the ease of removal of the dressing in contrast to conventional wound dressings treated with silver nitrate. Acticoat has shown an increase in antibacterial function over both silver nitrate and silver sulfadiazine. Acticoat is made up of nanocrystalline silver particles. Antibiotic-resistant strains have developed to both silver nitrate and silver sulfadiazine but not to nano-crystalline silver. The broader range of activity of nanocrystalline silver is apparently due to the release of both silver cations and uncharged silver species. Due to the continuing emergence of antibiotic resistant strains of infectious agents, a need exists for novel antibiotics.

Metal compounds have also played a significant role in other therapeutic applications. One example of the usefulness of the metals can be seen in the field of radiopharmaceuticals. The use of radiation therapy to destroy tumor cells is well known, but tumors can reappear after therapy. Hypoxic cells within the tumor are 2.5 to 3 times more resistant to X-ray radiation than other tumor cells. For this reason, these cells are more likely to survive radiation therapy or chemotherapy and lead to the reappearance of the tumor. Targeting of radionuclides to hypoxic cells will serve as a method to visualize them.

Complexes of γ -ray emitters such as ^{99}Tc are extremely useful as imaging agents, and therapeutic radiopharmaceuticals like ^{89}Sr , ^{153}Sm , ^{186}Re and ^{166}Ho are important in the treatment of bone tumors. Rh-105 emits a gamma ray of 319 keV (19%) that would allow in vivo tracking and dosimetry calculations. Many more radioactive nuclei can be harnessed by using the entire periodic table to construct diagnostic or therapeutic agents.

The usefulness of complexes of radioactive metals is highly dependent on the nature of the chelating ligand. A successful metal drug must both target a specific tissue or organ as well as rapidly clear from other tissues. In addition, for both imaging and tumor treatment, the target organ or tissue must have optimal exposure to the radiopharmaceutical. Therefore, there is a need for novel ligand systems designed to bind radioactive metals.

SUMMARY OF THE INVENTION

While metal complexes of some N-heterocyclic carbenes have been previously known, it has not been recognized that silver complexes of N-heterocyclic carbenes will act as antimicrobial agents. It has likewise not been recognized that complexes of N-heterocyclic carbenes and radioactive metals may be used as radiopharmaceuticals. Strongly chelating ligands, such as the pincer N-heterocyclic carbenes, described herein, can provide an alternate, more advantageous route for the generation of radiopharmaceutical complexes.

It is, therefore, an aspect of the present invention to provide a method of inhibiting microbial growth. The microbial growth is inhibited by exposing the microbe to a silver complex of a N-heterocyclic carbene.

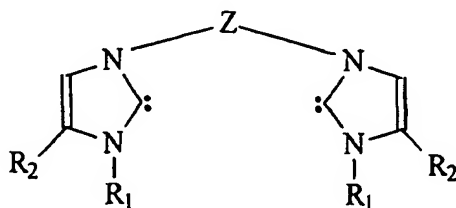
It is also an aspect of the present invention to provide a method of treating cancer cells. The cancer cells are treated by exposing them to a complex of a N-heterocyclic carbene and a radioactive metal. It is, therefore, also an aspect of the present invention to provide novel N-heterocyclic carbenes which, when complexed to silver, are useful as antimicrobial agents, and, when complexed to a radioactive metal, are useful as radiopharmaceuticals.

It is a further aspect of the present invention to provide method of synthesizing radiopharmaceuticals. It is also an aspect of the present invention to provide a method of synthesizing antimicrobial compounds.

At least one or more of the foregoing aspects, together with the advantages thereof over the known art relating to the treatment of infections, which shall become apparent from the specification which follows, are accomplished by the invention as herein after described and claimed.

In general, the present invention provides a method for inhibiting microbial growth or fungic growth comprising the step of administering an effective amount of a silver complex of an N-heterocyclic carbene.

The present invention also provides an N-heterocyclic carbene represented by the formula:



wherein Z is a heterocyclic group, and R₁ and R₂ are, independently or in combination, hydrogen or a C₁-C₁₂ organic group selected from the group consisting of alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, arylalkyl, alkylaryl, heterocyclic, alkoxy groups, and substituted derivatives thereof.

The present invention also provides a method for synthesizing a radiopharmaceutical compound comprising the steps of: reacting an imidazolium salt with either a transition-metal complex or a base to produce an N-heterocyclic carbene; and reacting the N-heterocyclic carbene with a metal to form a metal complex.

The present invention also provides a method for synthesizing an antibiotic compound comprising: reacting an imidazolium salt with a transition metal complex or a base to thereby produce an N-heterocyclic carbene; and reacting the N-heterocyclic carbene with a silver compound to thereby produce a silver complex with the N-heterocyclic carbene.

The present invention also provides a method for treating cancer cells comprising the step of administering an effective amount of a complex of an N-heterocyclic carbene and a radioactive metal.

The present invention also provides a method of creating an image of one or more tissues within a patient comprising the step of administering an effective amount of a complex of a N-heterocyclic carbene and a radioactive metal.

The present invention also provides a nanofiber comprising: a fiber-forming material; and a metal complex of an N-heterocyclic carbene.

The present invention also provides a radiopharmaceutical compound comprising a radioactive-metal complex of an N-heterocyclic carbene.

The present invention also provides a method for treating a cancerous tumor comprising the step of: administering an effective amount of a radioactive-metal complex of an N-heterocyclic carbene.

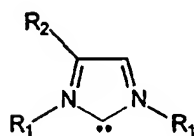
The present invention also provides a method of claim 28, wherein the radioactive metal is an element selected from the group consisting of transition metals, the lanthanide series, and the actinide series.

DETAILED DESCRIPTION OF THE INVENTION

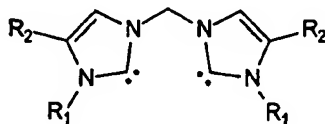
In this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. Unless defined otherwise, all

technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention pertains.

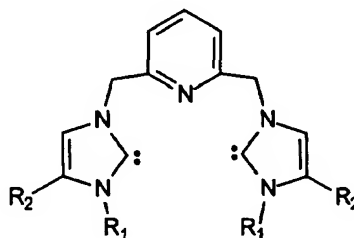
The present invention includes a metal complex of a N-heterocyclic carbene, its method of manufacture, and methods of use. Several general types of N-heterocyclic carbene ligands may be used as ligands for a metal such as silver. These include monodentate carbenes, such as those represented by formula 2, bidentate carbenes such as those represented by formulae 3-5, and bidentate macrocyclic carbenes such as those represented by formulae 6 and 7. With the exception of monodentate carbenes, each of these ligand types has as their basic constituent two N-heterocyclic carbene units bridged by either methylene groups, as in formula 3, dimethylpyridine groups, as in formula 4 and dimethylpyrrole groups as in formula 5, or are parts of rings as in formulae 6 and 7. The water solubility, stability, charge and lipophilicity of silver complexes of these N-heterocyclic carbenes may be modified by changes in R_1 and R_2 . Each R_1 and R_2 , separately or in combination, may be selected from the group consisting of hydrogen, C_1 - C_{12} alkyl, C_1 - C_{12} substituted alkyl, C_1 - C_{12} cyclo alkyl, C_1 - C_{12} substituted cycloalkyl, C_1 - C_{12} alkenyl, C_1 - C_{12} cycloalkenyl, C_1 - C_{12} substituted cycloalkenyl, C_1 - C_{12} alkynyl, C_1 - C_{12} aryl, C_1 - C_{12} substituted aryl, C_1 - C_{12} arylalkyl, C_1 - C_{12} alkylaryl, C_1 - C_{12} heterocyclic, C_1 - C_{12} substituted heterocyclic and C_1 - C_{12} alkoxy. It is particularly desirable, for at least some pharmaceutical applications, for R_1 and R_2 to be selected such that the resulting metal/N-heterocyclic carbene complex is soluble and stable in an aqueous solution.



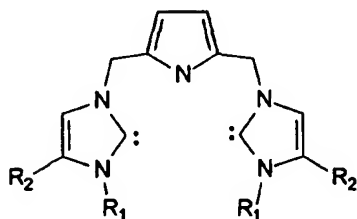
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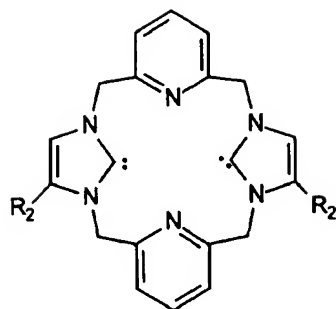
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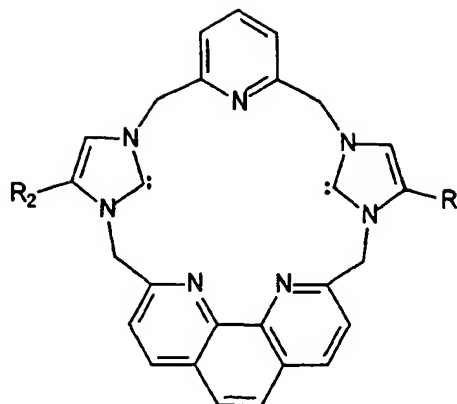
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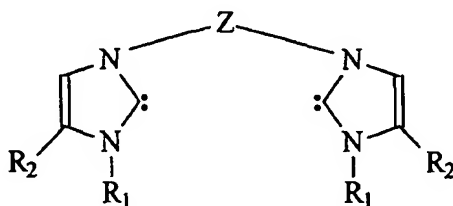
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7

In one example, the N-heterocyclic carbene is a bidentate carbene represented by formula 4 or 5, where R_1 is a C_1 - C_6 alkyl or C_1 - C_6 hydroxyalkyl group, and R_2 is a hydrogen atom. In one particular example, the N-heterocyclic carbene is represented by formula 4 or 5, where R_1 is a C_2 - C_3 hydroxyalkyl group, and R_2 is a hydrogen atom. In another example, the N-heterocyclic carbene is represented by formula 4 and each adjacent R_1 and R_2 together forms a substituted alkyl group.

As stated above, the present invention also provides novel N-heterocyclic carbenes represented by the formula



wherein Z is a heterocyclic group, and R_1 and R_2 are, independently or in combination, hydrogen or a C_1 - C_{12} organic group selected from the group consisting of alkyl, substituted alkyl, cyclo alkyl, substituted cycloalkyl, alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, aryl, substituted aryl, arylalkyl, alkylaryl, heterocyclic, substituted heterocyclic and alkoxy groups. In one example, Z is a pyridine or a pyrrole. In another example, Z is dimethylpyridine or dimethyl pyrrole.

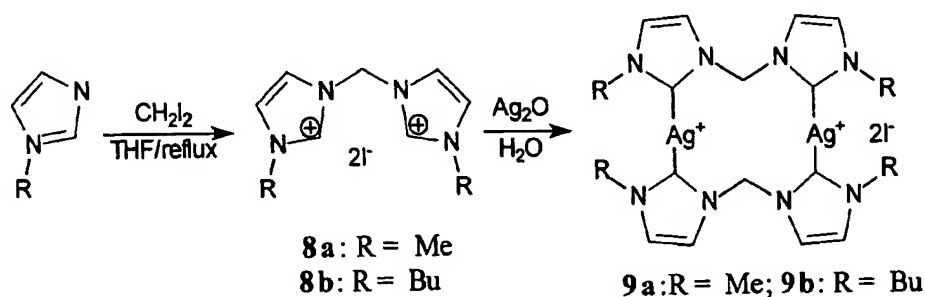
In general, imidazolium salts are the immediate precursors of N-heterocyclic carbenes. Several procedures may be used to convert imidazolium salts to the corresponding N-heterocyclic carbenes. N-Heterocyclic carbenes may be generated from imidazolium salts by deprotonation with bases such as $KOtBu$, KH , and NaH in solvents such as THF and liquid

ammonia. Isolatable N-heterocyclic carbenes may replace two-electron donors (such as tetrahydrofuran, carbon monoxide, nitriles, phosphines, and pyridine) on a variety of transition metal complexes to give N-heterocyclic carbene transition metal complexes. However it has not always been practical to isolate the carbenes.

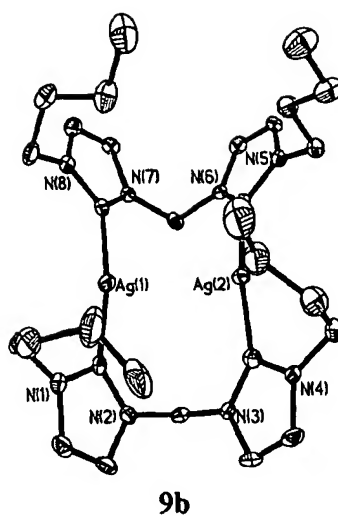
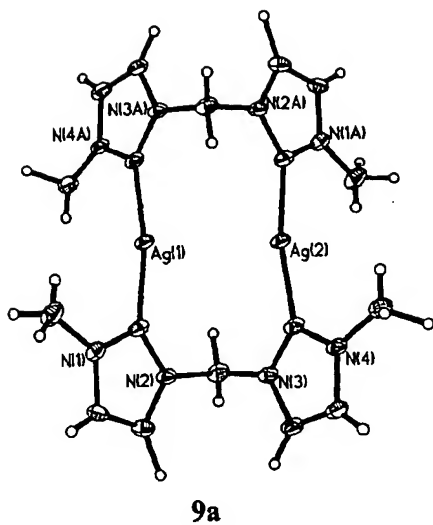
N-Heterocyclic carbene complexes may also be obtained by *in situ* generation of the N-heterocyclic carbene by deprotonation of the corresponding imidazolium salts in the presence of a suitable transition metal complex. Basic ligands on the metal complex, such as hydride, alkoxide, or acetate can deprotonate the imidazolium salt to form the N-heterocyclic carbene that readily binds to the vacant coordination site on a metal. For example $\text{Pd}(\text{OAc})_2$ has been shown to react with a variety of imidazolium salts to form palladium-carbene complexes.

The imidazolium salt can also be treated with an inorganic or organic base to generate the carbene. The reaction of imidazolium salts with metals containing basic substituents has been shown to be quite useful for the synthesis of transition metal complexes of carbenes. The combination of the basic oxide, Ag_2O , with imidazolium salts may be used to generate silver-carbene complexes. The use of silver-carbene complexes as carbene transfer reagents has been used to provide carbene complexes of gold(I) and palladium(II). Silver-carbene complexes have been employed in this manner to provide complexes with Pd-carbene and Cu-carbene bonds. The formation of transition metal-carbene bonds, using carbene transfer reagents is favored in many situations because the reactions proceed under mild conditions and without the use of strong bases.

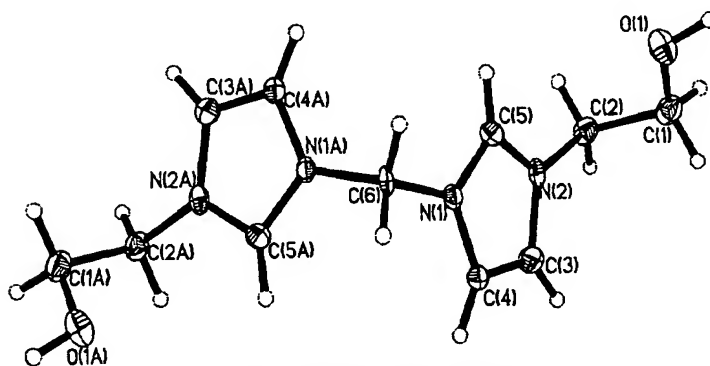
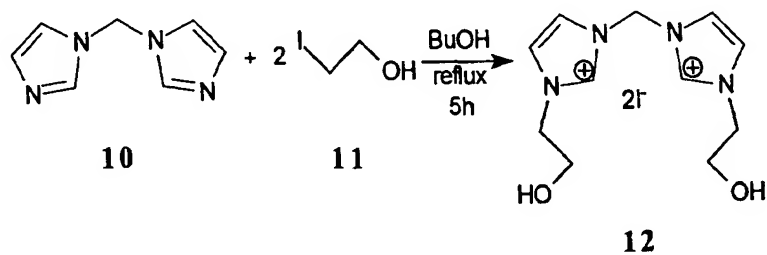
For example, the condensation of 2 equivalents of *n*-butyl imidazole or methyl imidazole and 1 equivalent of diiodomethane in refluxing THF affords the imidazolium salts shown as formulae 8a or 8b in high yield. The combination of shown as formulae 8a or 8b with Ag_2O in water forms the water soluble silver dimers 9a and 9b, respectively.



The thermal ellipsoid plots of the cationic portions of 9a and 9b are shown below.



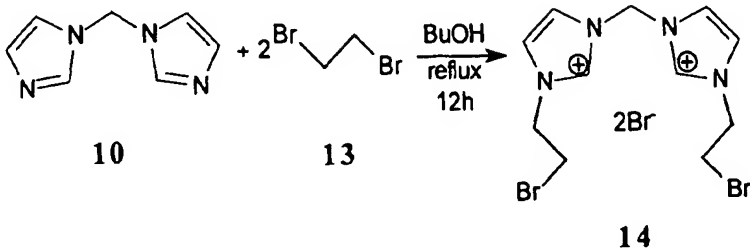
The combination of two equivalents of 1-iodoethanol (formula 11) with bisimidazol (formula 10) in refluxing butanol gives the water soluble diol shown as formula 12. This compound has been characterized by both NMR and X-ray crystallography.



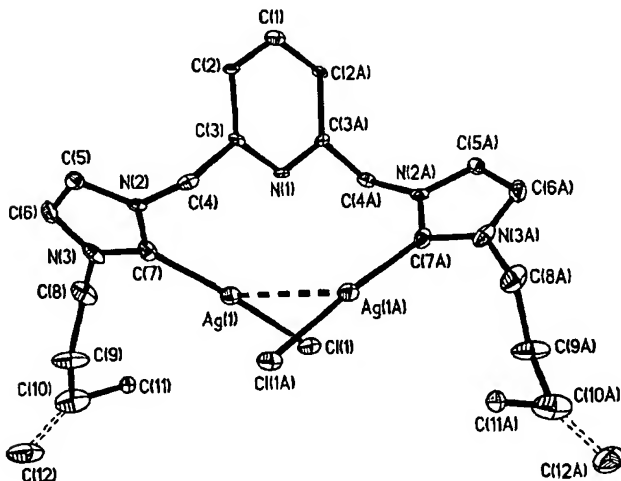
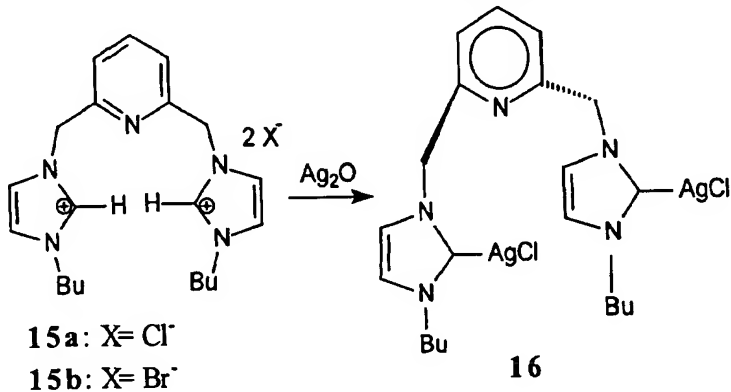
Thermal Ellipsoid Plot (TEP) of 12

A similar reaction has been carried out using 1,2-dibromoethane (formula 13) with bisimidazol to form the carbene represented by formula 14. The alcohol groups of compound 12

and the bromides of compound 14 provide functionalized sites for the incorporation of solubilizing moieties.

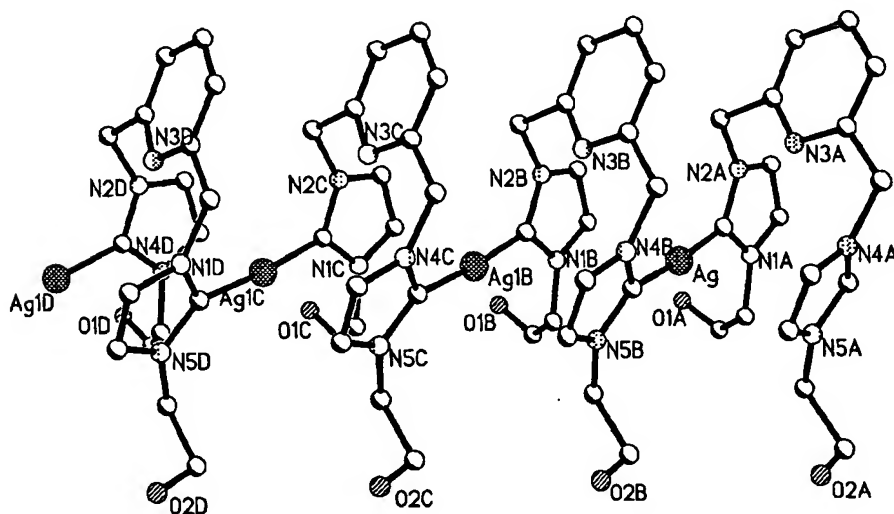
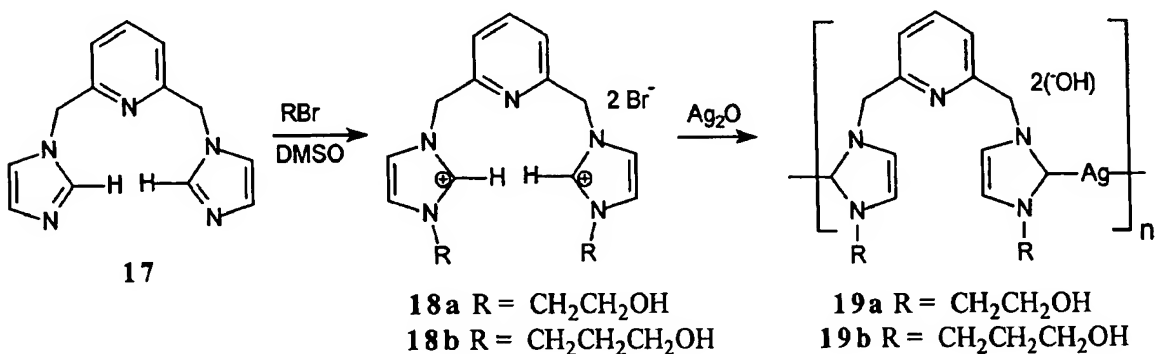


The pincer ligands 2,6-bis-(n-butylimidazoliummethyl)pyridine dihalide (compounds 15a and 15b) are easily obtained by the reaction of N-butyl imidazole with 2,6-bis(halogenmethyl)pyridine in a 2:1 molar ratio respectively. Ligand 15a readily reacts with Ag₂O in CH₂Cl₂ to yield the silver carbene complex 16. Complex 16 is stable in air and light.



TEP of 16

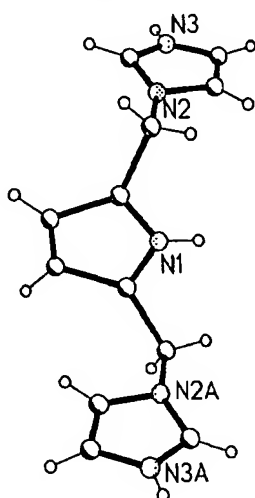
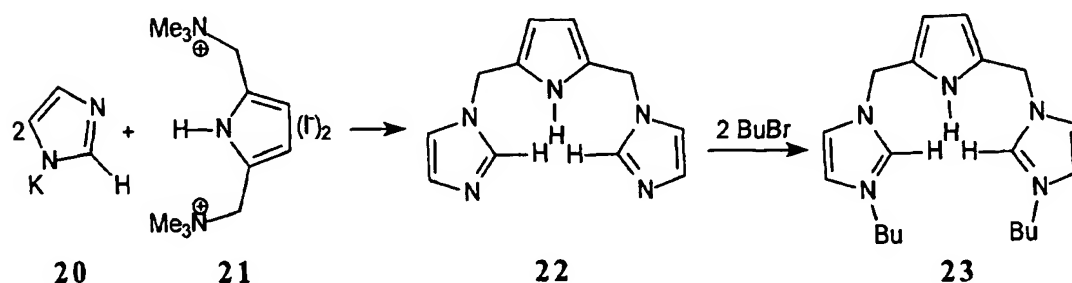
A general synthesis of pincer N-heterocyclic carbenes with a pyridine as the bridging unit is presented below. The reaction of two equivalents of potassium imidazole with 2,6-bis(bromomethyl)pyridine resulted in compound 18 in 70% yield. The combination of the compound represented by formula 17 with 2-bromoethanol or 3-bromopropanol gave 18a and 18b, respectively. The combination of the Br⁻ salt of 18a or 18b with an equimolar amount of Ag₂O gives the silver biscarbene polymers 19a and 19b, respectively. Compound 19a has been crystallographically characterized. The bromide salts represented by formulae 19a and 19b are very soluble and slowly decompose in water to give a silver mirror on the side of a flask containing either compound. 19a and its propanol analog 19b are effective antimicrobials. Derivatives of these complexes may be synthesized, using histidine as an example precursor as outlined below, to improve their antimicrobial properties.



TEP of 19a

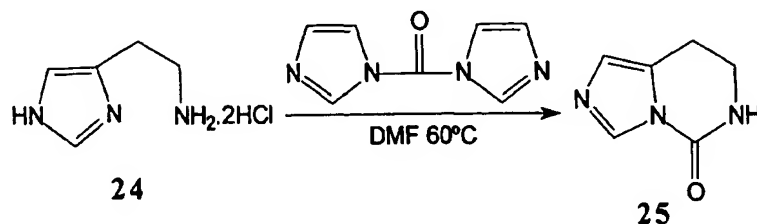
The combination of two equivalents of potassium imidazole (formula 20) with 2,5-bis(trimethylaminomethyl)pyrrole diiodide (formula 21) in THF gives compound 22. Compound

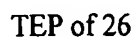
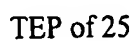
22 has been crystallographically characterized and its thermal ellipsoid plot is shown below. Addition of two equivalents of butyl bromide to compound 22 gives compound 23 in high yield.



TEP of 22.

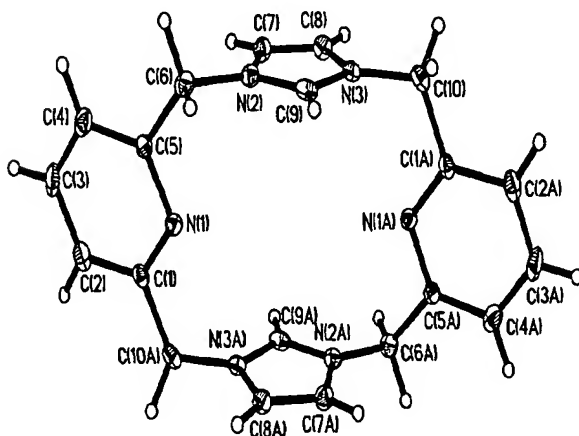
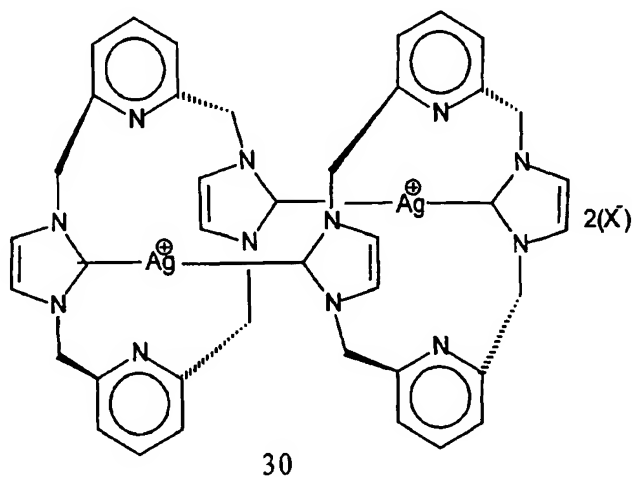
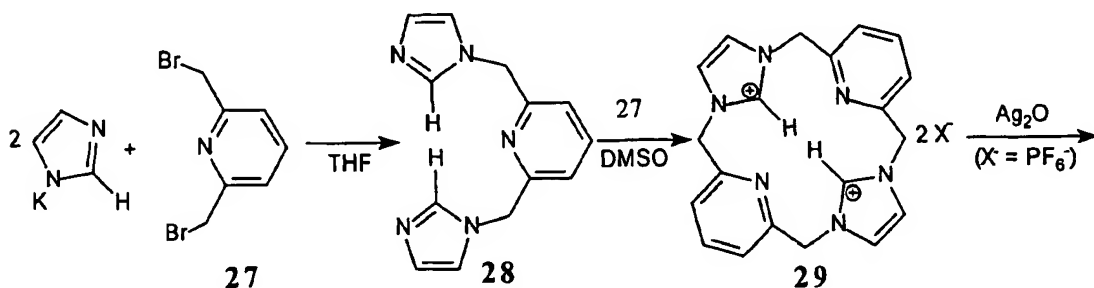
The reaction of histamine dihydrochloride (formula 24) with carbonyldiimidazole in DMF resulted in 5,6,7,8-tetrahydro-5-oxoimidazo [1,5-c]pyrimidine (formula 25) in 40% yield. The compound of formula 25 has been crystallographically characterized (see thermal ellipsoid plot below). The combination of two equivalents of compound 25 with one equivalent of 2,6-bis(bromomethyl)pyridine in acetonitrile resulted in the formation of compound 26 in very high yield.



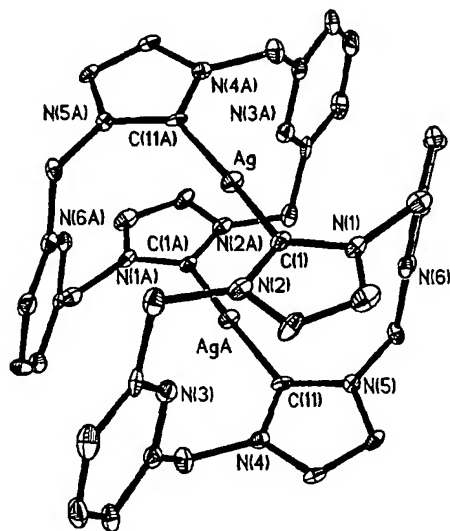


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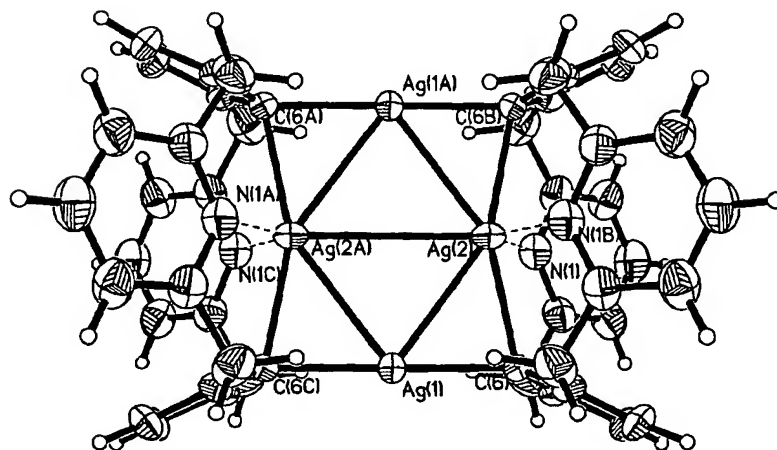
of the PF_6^- salt of compound 29 with an equimolar amount of Ag_2O gives a silver biscarbene dimer (formula 30) in nearly quantitative yield. Compounds 29 and 30 have been crystallographically characterized. The bromide salt of compound 30 ($\text{X}=\text{Br}$), is soluble and stable in water. Under analogous reaction conditions, the combination of compound 29 with 4 equivalents of Ag_2O gives a tetra-silver biscarbene dimer (formula 31). The combination of compound 29 ($\text{X}^- = \text{Br}^-$) with Ag_2O in water directly gives the bromide salt of compound 30. Halide salts of compound 30 can be synthesized in water, and are water soluble. The bromide and chloride salts of compound 30 are effective antimicrobials.



TEP of dicationic portion of compound 29[PF₆]⁻₂

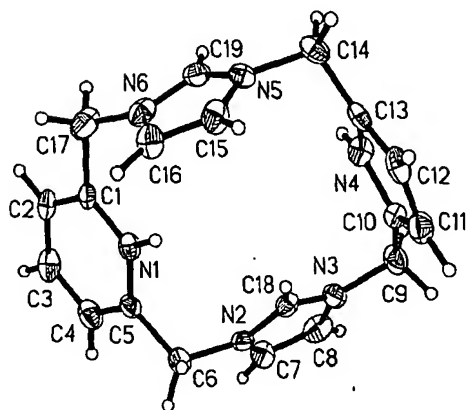
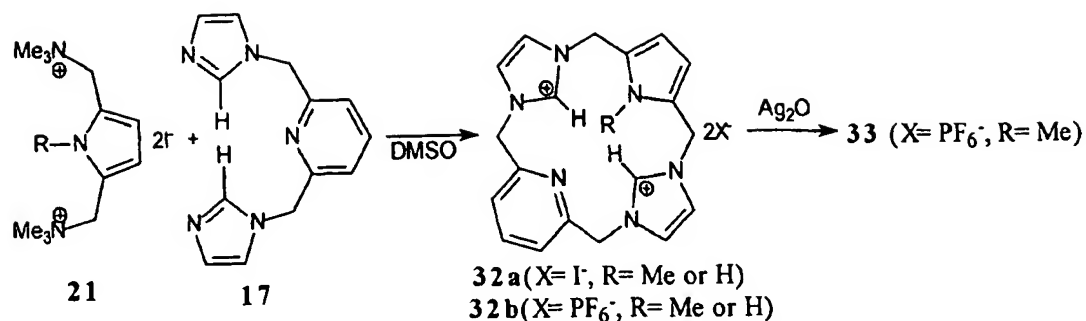


TEP of dicationic portion of compound 30[PF₆]⁻₂

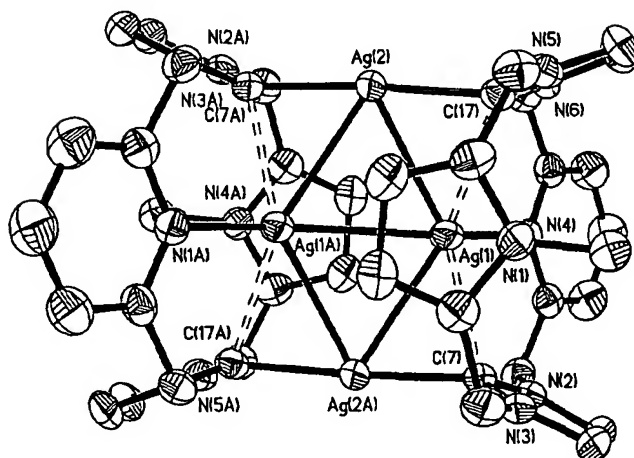


TEP of tetracationic portion of compound 31[PF₆]⁻₄

The 3 + 1 condensation of the pyrrole shown by formula 21 (R=H or Me), with the pyridine shown by formula 17 gives the compound of formula 32 (R=H or Me). Anion exchange of 32a with NH₄⁺PF₆⁻ gives compound 32b. The combination of 32b (X=PF₆⁻, R=Me) with four equivalents of Ag₂O gives a tetra-silver biscarbene dimer, compound 33 (X=PF₆⁻, R=Me), the thermal ellipsoid plot of which is shown below.

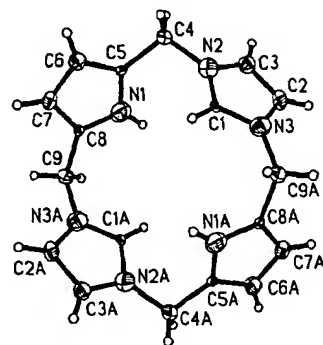


TEP of compound 32b ($X=PF_6^-$, $R=H$).

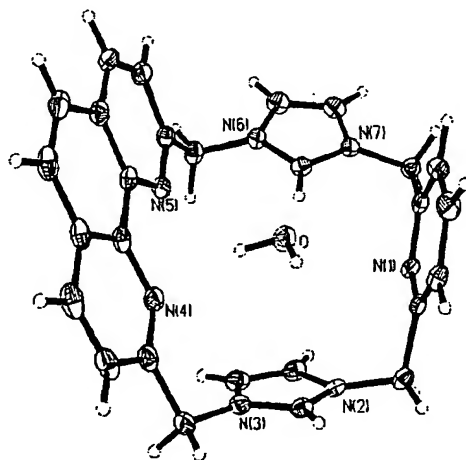


TEP of tetracationic portion of compound 33[PF_6^-]₄

Addition of one equivalent of compound 21 to compound 22 gives the bisimidazolium porphyrinoid 34 in high yield and on a large scale. Compound 34 has been crystallographically characterized and the thermal ellipsoid plot of the dication ring of 34 is shown below. The combination of compounds 32 ($R=H$) and 34 with 4 equivalents of Ag_2O affords tetra-silver biscarbene dimers analogous to compounds 31 and 33.

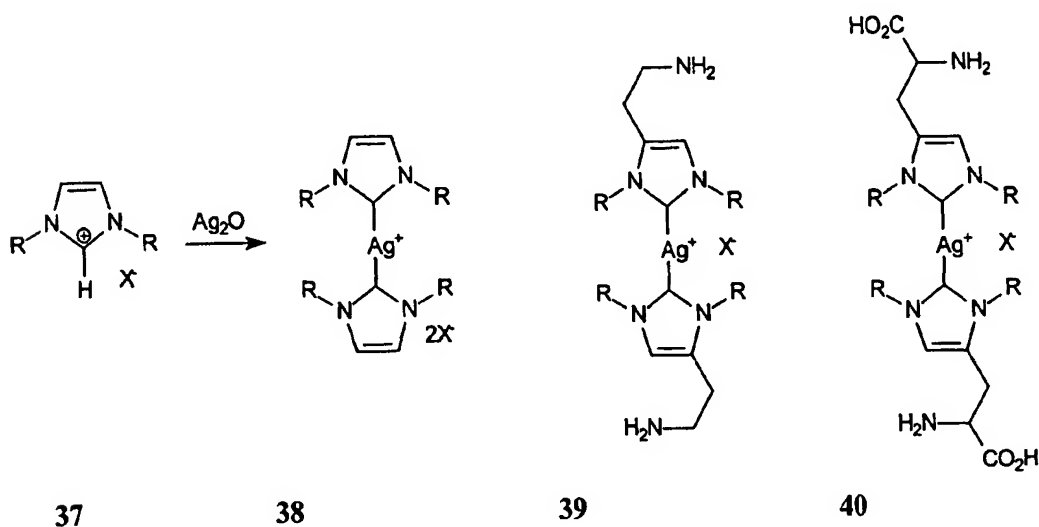


The reaction scheme shows the synthesis of macrocyclic receptor **36** from the starting materials **17** and **35**.
17 is 1,3-bis(2-pyridylmethyl)imidazole, consisting of an imidazole ring connected at its 1 and 3 positions to two 2-pyridylmethyl groups.
35 is 1,3-bis(bromomethyl)anthracene, consisting of an anthracene ring with bromomethyl groups at the 1 and 3 positions.
The reaction is a macrocyclization where the pyridine rings of **17** coordinate with the bromomethyl groups of **35** to form a large macrocyclic ring. The product **36** is a macrocyclic receptor with a central cavity, featuring two imidazole rings and two anthracene units linked by pyridine rings. The imidazole rings are shown with a positive charge on one of the nitrogen atoms, indicating they are part of a cationic receptor.



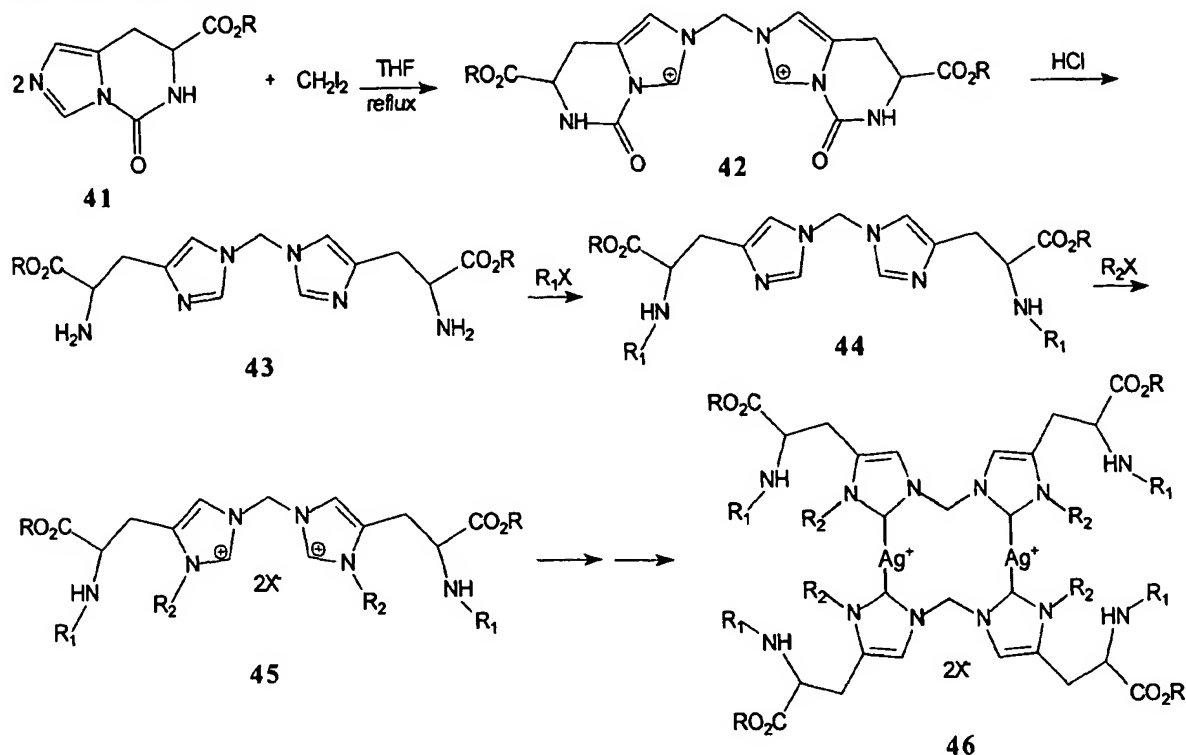
TEP of compound 36

Monodentate N-heterocyclic carbene silver complexes such as those represented by formula 38 may be synthesized by the interaction of the imidazolium precursors 37 with silver oxide. As mentioned above, the side chains, R, may be chosen so as to modify the water solubility, lipophilicity and other properties of the complexes. For example, R may be hydrogen or a C₁-C₁₂ organic group selected from the group consisting of alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, arylalkyl, alkylaryl, heterocyclic, and alkoxy groups and substituted derivatives thereof. Silver complexes such as those represented by formulae 39 and 40, synthesized from histamine and histidine, respectively, may be synthesized and used as antimicrobial compounds. Because histamine and histidine are present in the body, their derivatives are expected to give the least skin irritation when used as a topical antimicrobial and to provide very limited problems as an internal antimicrobial with excellent toxicological properties.

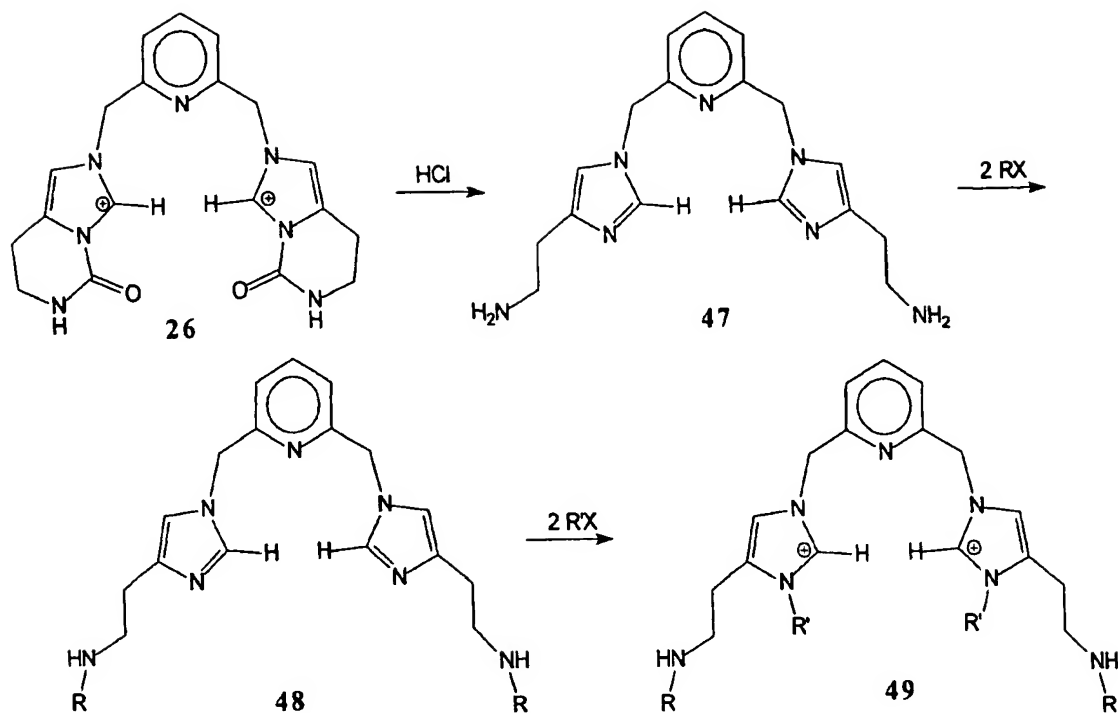


The synthesis of the pincer N-heterocyclic carbenes having methene or methylene groups bridging the two N-heterocyclic carbenes (see formula 3) and with substituents attached is provided below. The substituents may be chosen in order to give the overall complex sufficient solubility, lipophilicity or other properties. Pyridine rings and imidazoles serve as the fundamental building blocks in the procedures discussed below. Based on the synthesis of compounds 8a and 8b above, two equivalents of compound 41 will combine with methylene iodide to form compound 42. Opening of compound 42 with HCl will provide compound 43. One equivalent of an alkyl halide would readily add to the primary amines of compound 43, because primary amines are more reactive than imidazole nitrogens, to form compound 44. A second alkyl halide would add to the secondary imidazole nitrogens of compound 44 to form the bisimidazolium cation shown as

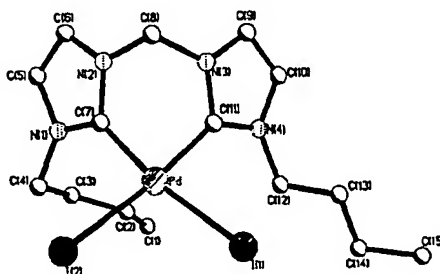
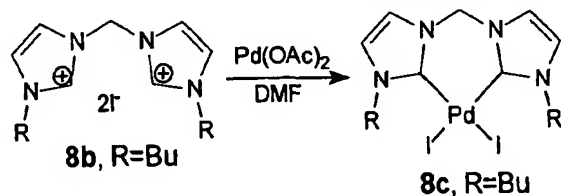
compound 45. The *bis*imidazolium cation 45 may be combined with Ag₂O to form silver complexes shown as formula 46 similar to compounds 9a and 9b above.



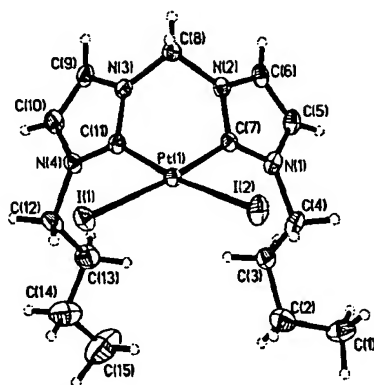
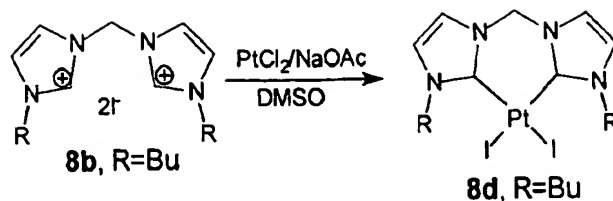
Compound 26 may be treated with HCl to give compound 47, which may then be contacted with a derivatized alkyl halide containing a solubilizing substituent to give compound 48. Compound 47 could also be derivatized with a carboxylic acid and dicyclohexylcarbodiimide (DCC) to form an amide bond. The combination of compound 48 at a higher temperature with a derivatized alkyl halide that similarly contains a solubilizing substituent will give the imidazolium biscation shown as formula 49, which may be further complexed with metals such as rhodium.



Silver-carbene complexes may also be used as carbene transfer reagents to create other carbene complexes. The formation of transition metal-carbene bonds, using carbene transfer reagents is favored in many situations because the reactions proceed under mild conditions and without the use of strong bases. For example, the combination of 8b with $\text{Pd}(\text{OAc})_2$ in DMF followed by treatment with NaI in acetonitrile results in the formation of the compound represented by formula 8c. The thermal ellipsoid plot of this compound is shown below. Similarly, the combination of 8b with PtCl_2 and sodium acetate in DMSO gives the compound represented by formula 8d in 50% yield. The X-ray thermal ellipsoid plot of 8d is shown below.

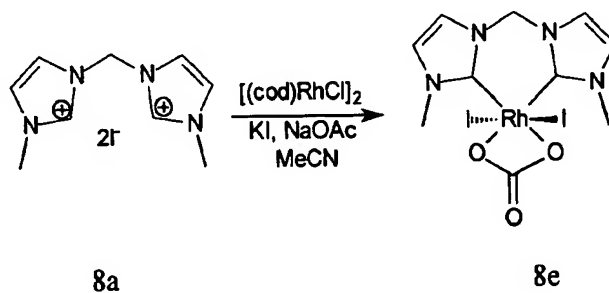


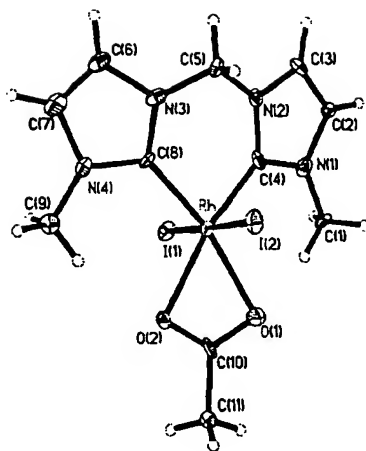
TEP of 8c



TEP of 8d

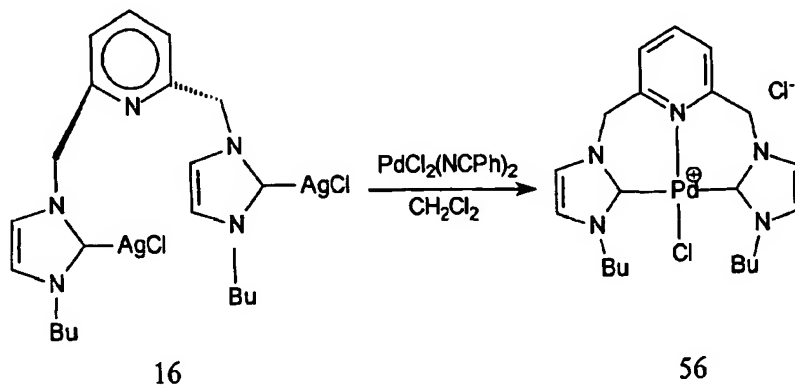
The combination of the imidazolium salt represented by formula 8a with [(1,5-cyclooctadiene)RhCl]₂ in refluxing MeCN in the presence of NaOAc and KI gives the rhodium carbene 8e in 80% yield. This compound has been characterized by ¹H and ¹³C NMR and X-ray crystallography. This rhodium complex is water stable for extended periods of time. A related chelating *bis*-carbene rhodium complex has been synthesized and has been shown to be stable enough to use in catalytic processes.



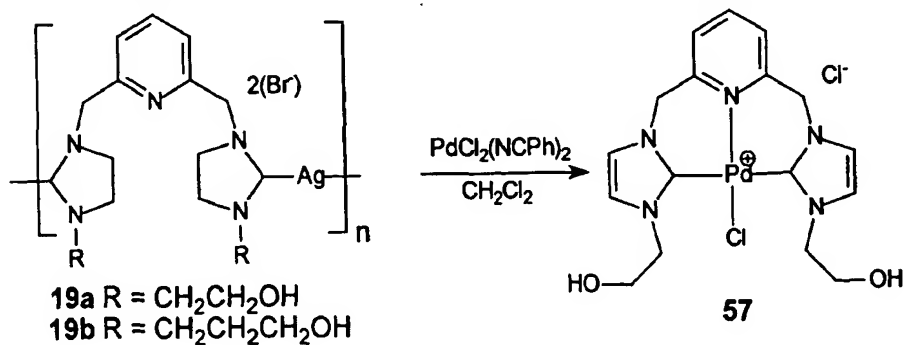


TEP of 8e

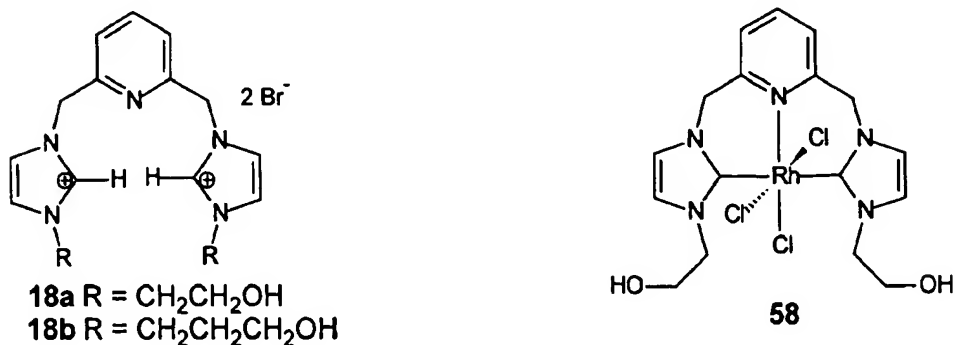
The silver complex of an N-heterocyclic carbene represented by formula 16 can function as a carbene transfer reagent. The reaction of complex 16 with $(\text{PhCN})_2\text{PdCl}_2$ in CH_2Cl_2 yields the palladium carbene complex represented by formula 56 and two equivalents of AgCl in nearly quantitative yield.



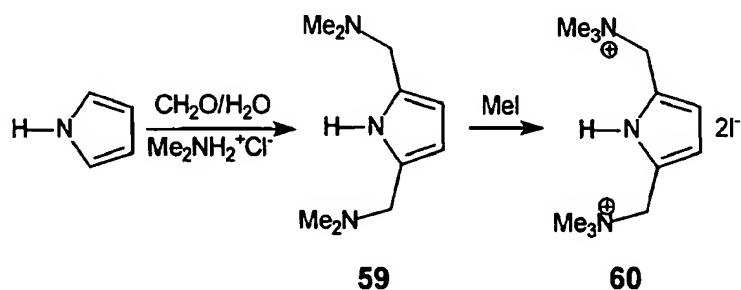
Similarly, the reaction of the complex represented by formula 19a with $(\text{PhCN})_2\text{PdCl}_2$ in CH_2Cl_2 yields the palladium carbene complex represented by formula 57.



A similar synthesis route may be used to synthesize the compound represented by formula 58 from the compound represented by formula 18a.

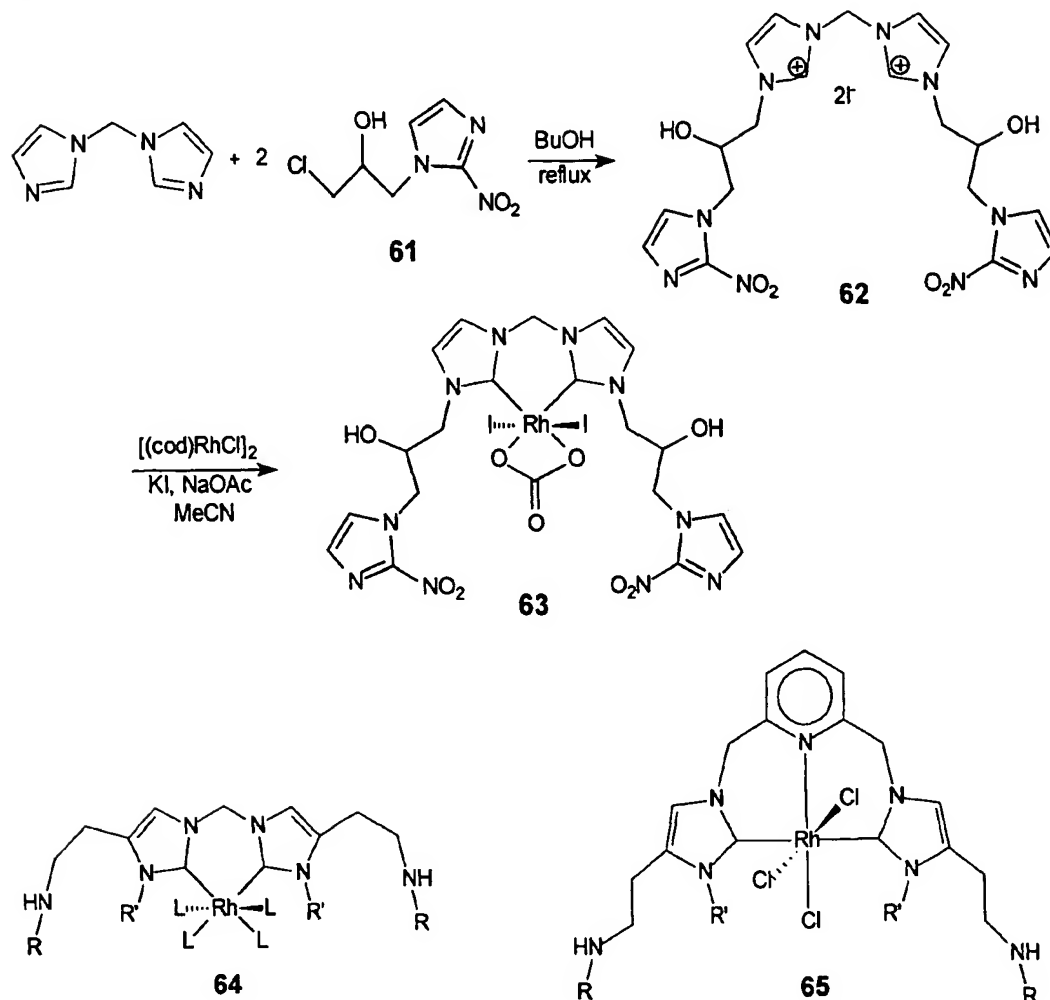


For the synthesis of pyrrole bridged pincer N-heterocyclic carbenes, a 2,5-bisdimethylpyrrole with leaving groups on the methyl groups is particularly useful in the synthesis method of the present invention. The Mannich reaction of dimethylammonium chloride in aqueous formaldehyde and pyrrole gives 2,5-bisdimethylaminomethylpyrrole, represented by formula 59. Addition of iodomethane to pyrrole 59 in THF gives 2,5-*bis*(trimethylaminomethyl)pyrrole diiodide (formula 60).



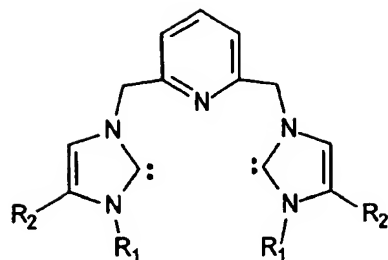
A molecule containing a 2-nitroimidazole group is believed to be targeted to hypoxic cells. These compounds are reduced at the nitroimidazole group and trapped within cells with a low oxygen environment. Attachment of a 2-nitroimidazole group to pincer N-heterocyclic carbenes to form the compound represented by formula 62 may be accomplished as follows. The condensation of the compound represented by formula 61 with bisimidazol in a 2:1 ratio is expected to give the compound represented by formula 62. Other derivatives of 2-nitroimidazole having various linker segments may similarly be synthesized. The variety of linker groups, including polyethylene oxide (PEO), will allow for flexibility in positioning the chelator relative to the targeting group as well as for variation of the octanol/water partition coefficient of the compound, which is relevant to the clearance through the kidneys. The formation of rhodium complexes similar to 62 is also

envisioned. Similar procedures may be used to synthesize derivatives of 64 and 65 containing nitroimidazole and solubilizing substituents.



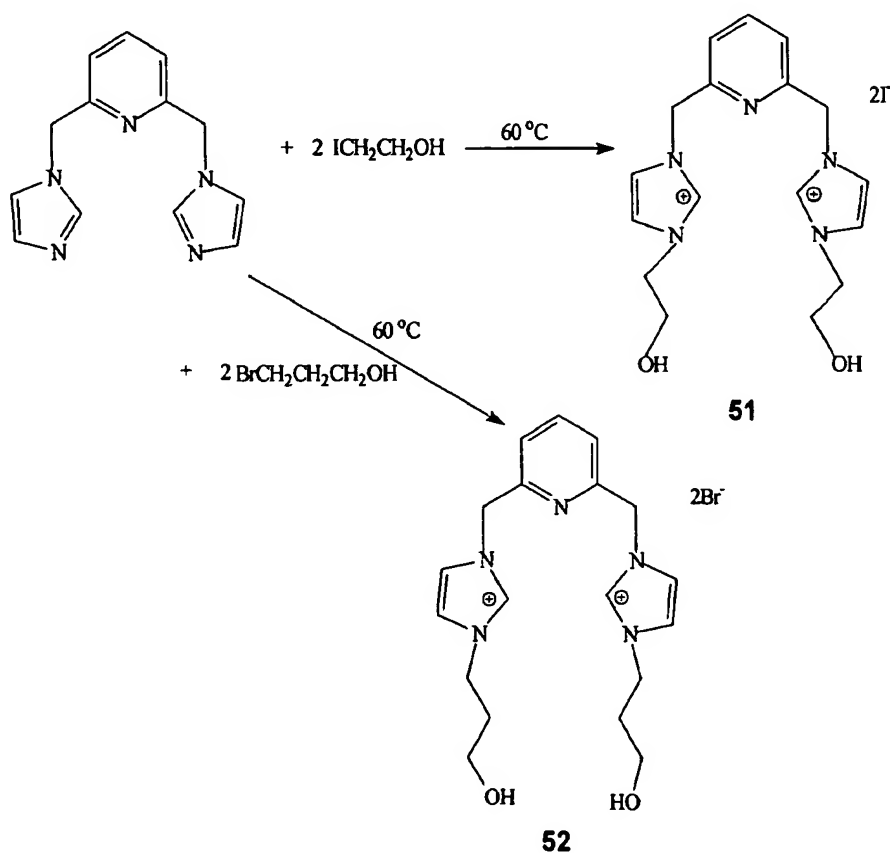
Isotopes of the metals indicated herein as components of an N-heterocyclic carbene complex may be used to form radiopharmaceuticals. For example, ¹⁰⁵Rh may be used in place of Rh. ¹⁰⁵Rh has a convenient half-life of 1.5 days and also emits relatively low levels of γ -radiation. This isotope of rhodium decomposes by beta emission to ¹⁰⁵Pd a stable naturally occurring isotope of palladium. Other employable isotopes can be selected from transition metals, elements from the lanthanide series, and elements from the actinide series. Preferred isotopes are Ag, Rh, Ga, and Tc.

In order to demonstrate the practice of the present invention, two N-heterocyclic carbenes were synthesized and tested for antimicrobial properties as described below. The compounds can be shown with reference to formula 4

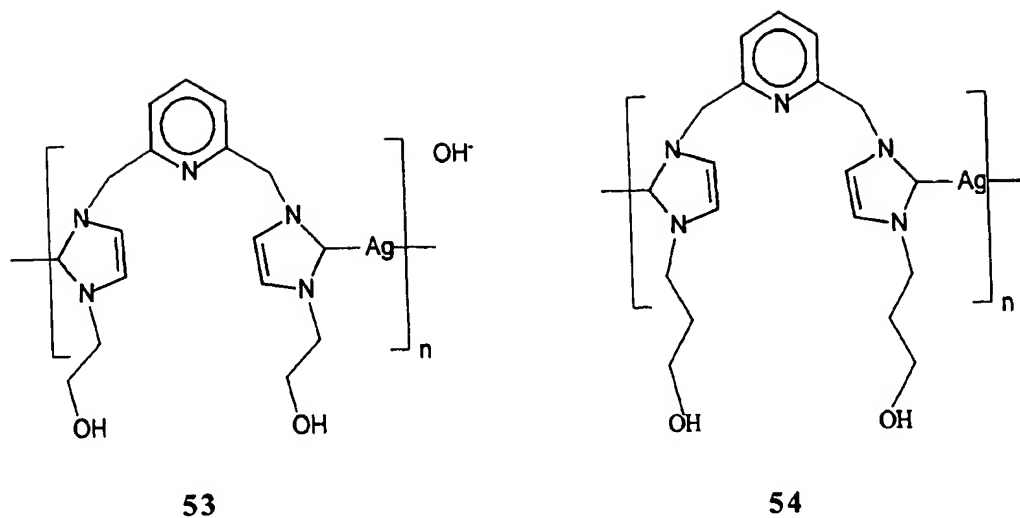


4

where R_1 is a hydroxyethyl or hydroxypropyl group and R_2 is a hydrogen atom. These carbenes were synthesized by reacting 2,6-bis-(imidazolmethyl)pyridine with either 2-iodoethanol or 3-bromopropanol to provide compounds of formulas 51 and 52.



The IR spectra for these compounds show an O-H stretching band vibration, 3325 cm^{-1} . FAB-MS spectra obtained from these compounds in nitrobenzyl matrices showed $[51][I]^+$ ($C_{17}H_{23}N_5O_2I$) at m/z 456 and $[52][I]^+$ ($C_{19}H_{27}N_5O_2Br$) at m/z 436. These compounds readily react with Ag_2O to form the silver-bis(carbene) pincer complexes 53 and 54 in high yield.



The formation of compounds 53 and 54 is confirmed by the loss of the imidazolium proton at 9.13 ppm, 9.36 ppm in the ^1H NMR spectra of these compounds, and the appearance of a resonance at 181 ppm in the ^{13}C NMR spectra of these compounds. Further evidence for the formation and structure of compound 53 is provided by X-ray crystallography.

Colorless crystals of compound 53 were obtained by slow evaporation of a methanol solution of compound 53. Interestingly, compound 53 undergoes complete anion exchange in aqueous methanol, replacing the iodide anions with hydroxide anions. In the solid state, compound 53 exists as a one-dimensional linear polymer as shown in Fig. 1. Fig. 1 is a thermal ellipsoid plot of compound 53 with the thermal ellipsoid drawn at a 30 percent probability level. The hydrogen atoms have been omitted from Fig. 1 for clarity.

The geometry at the silver atoms is nearly linear with a C5-Ag1-C15 bond angle of $174.7(4)^\circ$, and Ag1-C5, and Ag1-C15 bond distances of $2.108(11)$ Å and $2.060(13)$ Å, respectively. Mass spectroscopy suggests that in solution and in the gas phase, compound 53 exists as monomer, whereas X-ray crystallography shows that compound 53 is polymeric in the crystal.

An anion exchange reaction of compound 53 with aqueous ammonium hexafluorophosphate, results in the formation of compound 55. In the solid state, compound 55 exists as a dimer, as shown in Fig. 2. Fig. 2 is a thermal ellipsoid plot of compound 55 with the thermal ellipsoid drawn at a 30 percent probability level. The hydrogen atoms have been omitted from Fig. 2 for clarity. The geometry of the silver atoms are nearly linear with C32-Ag1-C5 ($175.7(4)^\circ$), C22-Ag2-C17 ($174.6(3)^\circ$) bonds angles, and Ag1-C32 ($2.070(9)$ Å), Ag1-C5 ($2.091(9)$ Å), Ag2-C22 ($2.064(9)$ Å), Ag2-C17 ($2.074(8)$ Å) bond lengths. The nature of the anions is significant to the structural changes of compound 53 versus compound 55, and the choice of anion

has a pronounced effect on the solubility of these compounds. For example, compound 53 is soluble in aqueous media whereas compound 55 is not. Table 1 gives a summary of the crystal data of both of these compounds.

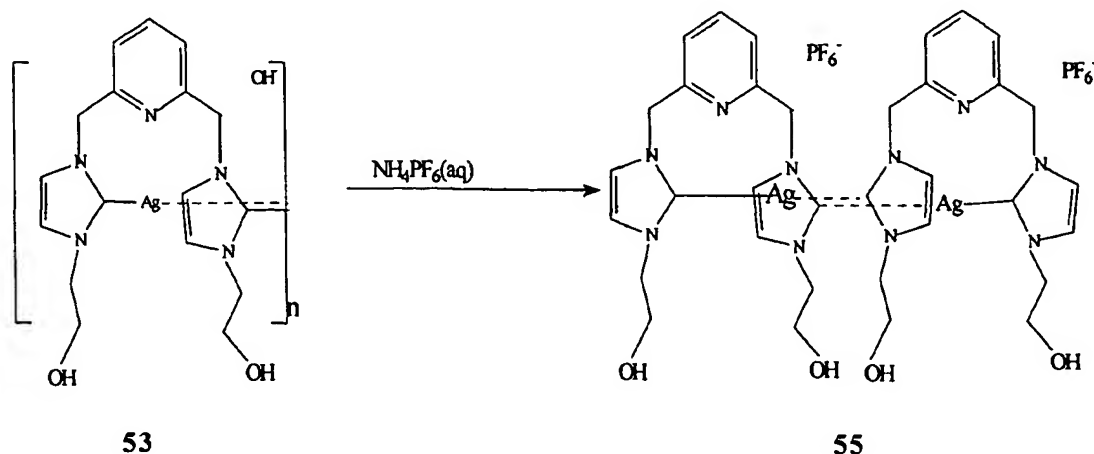


Table 1.

Empirical Formula	53 , C ₁₇ H ₂₂ N ₅ O ₃ Ag	55 , C ₃₄ H ₄₂ N ₁₀ O ₄ AgP ₂ F ₁₂
Formular Weight	434.0735	868.1481
Temperature (K)	100	100
Wavelength (Å)	0.71073	0.71073
Crystal system, space group, Z	Orthorhombic, P2(1)2(1)2(1), 4	Monoclinic, P2(1)/c, 8
Unit cell dimensions		
a (Å)	4.5586(17)	10.9448(14)
b (Å)	14.900(6)	22.885(3)
c (Å)	29.923(12)	17.729(2)
α (°)	90	90
β (°)	90	92.196(2)
γ (°)	90	90
V (Å ³)	2032.5(14)	4437.4(10)
Dcalc (Mg/m ³)	1.422	1.737
Absorption coefficient (mm ⁻¹)	1.010	1.055
Theta range for data collection (°)	1.36 to 24.99	1.45 to 25.00
Reflections collected/unique	6300/3506 [R(int) = 0.0650]	20811/7757 [R(int) = 0.0437]
Goodness-of-fit on F ²	1.034	1.058
Final R indices[I > 2 σ (I)]	0.0655	0.0956
R indices (all data)	0.1410	0.2491
Largest difference peak and hole (e Å ⁻³)	0.954 and -0.875	2.069 and -1.230

The usefulness of compounds 53 and 55 as antimicrobial agents was evaluated. The standard agar plates overlay method was used to obtain the sensitivity data as presented in Table 2. In this test, a filter paper disc of 6mm diameter was soaked with 20 μ L of a silver compound of known concentration, and placed over a lawn of an organism in the agar plate. The diameter of the area in which growth of the organism is inhibited by the test solution was measured after an overnight incubation as a measure of the relative antimicrobial activity of the silver compounds. The test organisms were *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Silver nitrate was the reference standard used, while compounds 51 and 52 served as a negative controls.

Table 2.
Antimicrobial Activity of Silver Compounds

Tested compounds	Ag ⁺ (ug/ml)	Diameter of Inhibited Area (mm)		
		<i>E. coli</i>	<i>S. aureus</i>	<i>P.aeruginosa</i>
AgNO ₃	3176	11.38	10.88	11
0.5%(w/v)				
53	3130	11.5	11	12
1.31%				
55	3195	11.58	10.67	10.25
1.42%				
53	1195	10.13	10	11.13
0.50%				
55	1125	10	9	12
0.50%				
51		6	6	6
0.50%				
52		6	6	6
0.50%				

The data confirmed that compounds 53 and 55 have antimicrobial properties at a level comparable to silver nitrate as shown in Table 2. The pincer ligands, compounds 51 and 52, were found to have no antimicrobial activity.

The silver compounds were also tested according to the minimum inhibition concentration determination method (MIC). The MIC is a standard microbiological technique used to evaluate the bacteriostatic activity of antimicrobial agents. In this case, the MIC was based on the total amount of silver available and not on the concentration of silver ions. A 0.5 percent (w/v) solution of each of the silver compounds 53 and 55 was tested. On dissolving of the silver complexes in the culture medium (LB broth), a precipitate of AgCl was observed in all samples. The activity of a dilution series of the supernatant portion of the silver complex solutions was evaluated, with the addition of a constant volume of freshly grown organism (20µl) per day. *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were again used as the test organisms. The MIC was obtained by visual inspection of the cultures for growth(+) or no growth(-) as reported in Table 3. In Table 3, DF is the dilution factor. From the results, it can be concluded that compounds 53 and 55 are less bound to chloride ion than silver nitrate, due to the stability of the Ag-C donor ligand bond. Thus, compounds 53 and 55 show better antimicrobial activity than silver nitrate. This is a desirable property of compounds 53 and 55, when considering silver compounds for in vivo application. It may be noted that although equal weights of silver compounds were used, the amount of silver ions released by compounds 53 and 55 is about 2.7 times lower than the amount of silver ions released by silver nitrate.

Table 3.
MIC Results of Supernatants of Silver Compounds (less silver chloride)

Test Ag compounds	E. coli		P. aeruginosa		S. aureus		
	Ag (ul/ml)	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
53	1186	-	-	-	-	-	-
x 1DF		-	+	-	-	-	+
x 2DF		-	+	-	+	+	
x 3DF		+		+		+	
x 4DF		+		+		+	
55	1125	-	-	-	-	-	-
x 1DF		-	+	-	+	-	+
x 2DF		-	+	-	+	+	
x 3DF		+		+		+	
x 4DF		+		+		+	
AgN03	3176	-	+	-	+	+	
x 1DF		+		+		+	
x 2DF		+		+		+	
x 3DF		+		+		+	
x 4DF		+		+		+	

While not wishing to condition patentability on any particular theory, it is believed that the activity and stability of compounds 53 and 55, as well as their solubility in water, may be attributed to the relatively slow decomposition of Ag-C donor ligand bond over time to silver metal and silver ion.

When the MIC test was repeated as described above except in the presence of insoluble silver chloride, the activity of the silver compounds was enhanced, with silver nitrate performing better as shown in table 4. It has been previously reported that the presence of chloride contributes to the toxicity of silver in sensitive strains of organisms.

Table 4.

Effect of chloride (as silver chloride) in the bacteriocidal activity of the silver compounds

Tested Ag compounds (% w/v)	<i>E. coli</i> (Days)						<i>P. aeruginosa</i> (Days)						<i>S. aureus</i> (Days)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
53																		
0.50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.03	-	-	+				-	-	+				-	+				
55																		
0.50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.03	-	-	+				-	-	+				-	+				
AgNO₃																		
0.50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.03	-	-	-	+			-	-	+				-	-	+			

The minimum lethal concentration was determined to evaluate the bacteriocidal properties of the compounds represented by formulae 53 and 55. The clear (no growth) portion of the culture media with the lowest Ag compound concentration was used, by streaking 0.01 ml of the solution on agar plate using a sterilized loop followed by incubation at 37 °C for 24 – 48 hours. The colonies were visually counted, with the end point of the minimum bacteriocidal concentration (MBC) as no growth on the agar plate. The test compounds showed an improved bacteriocidal effect compared to silver nitrate up to the seventh day of incubation and MBC test, with no growth observed after the tenth day of incubation and testing for the silver compounds. This is despite the fact that freshly grown organisms were added each day to the culture media containing the silver compounds throughout the incubation period. The bacteriocidal and bacteriostatic properties of 53 and 55 are believed to be due to the slow decomposition of the Ag-C donor (carbene) ligand bond over time to silver metal, silver ion, AgCl and to their solubility in water.

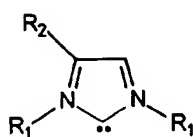
The alkanol N-functionalized silver carbene complexes 53 and 55 are soluble in aqueous media. In addition, they have proved to be useful antimicrobial agents, and their solubility in water makes them excellent silver compounds that can be of use for *in vivo* application. The solubility and stability of silver complexes in chloride solution have been key factors that have limited the use of silver complexes for *in vivo* application.

It should be evident that the present invention is highly effective in providing a method of inhibiting microbial growth by administration of a N-functionalized silver carbene complex. It is, therefore, to be understood that any variations evident fall within the scope of the claimed invention and thus, the selection of specific component elements can be determined without departing from the spirit of the invention herein disclosed and described.

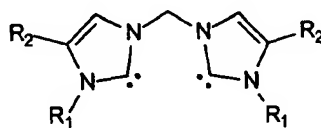
CLAIMS

We claim:

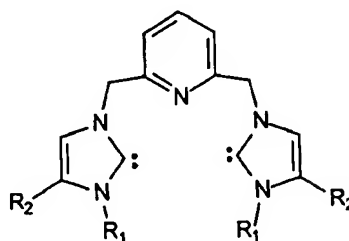
1. A method for inhibiting microbial growth or fungic growth comprising the step of administering an effective amount of a silver complex of an N-heterocyclic carbene.
2. The method of claim 1, wherein the N-heterocyclic carbene is selected from the group consisting of compounds represented by formulae 2-7:



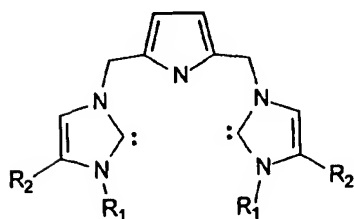
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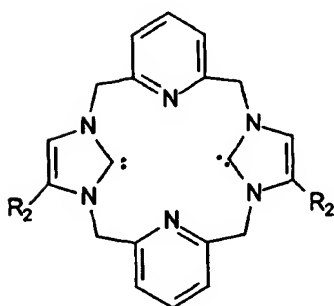
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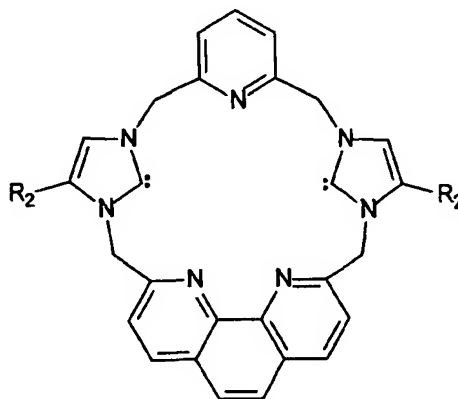
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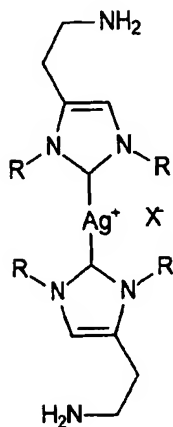
6



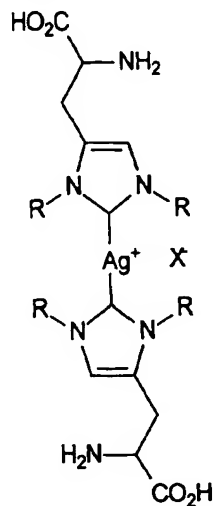
7

wherein R_1 and R_2 are, independently or in combination, hydrogen or a C_1 - C_{12} organic group selected from the group consisting of alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, aryl, substituted aryl, arylalkyl, alkylaryl, pyrroles, pyridines, thiophenes and alkoxy.

3. The method of claim 1, wherein the silver complex of a N-heterocyclic carbene is selected from the group consisting of compounds represented by formulae 39 and 40:



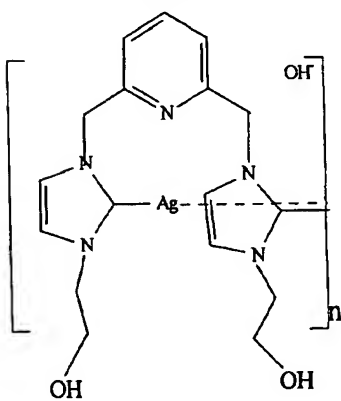
39



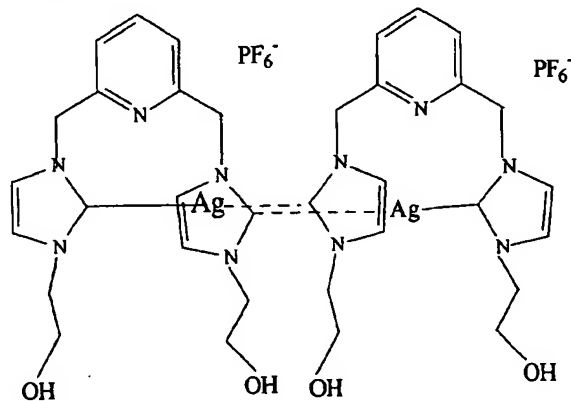
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wherein R is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, arylalkyl, alkylaryl, heterocyclic, and alkoxy groups and substituted derivatives thereof, and X is an anion.

4. The method of claim 1, wherein the silver complex of an N-heterocyclic carbene is selected from the group consisting of compounds represented by formulae 53 and 55:

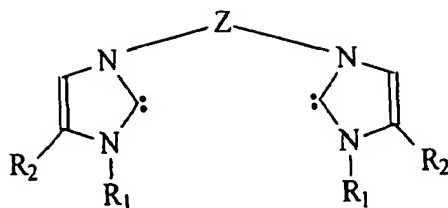


53



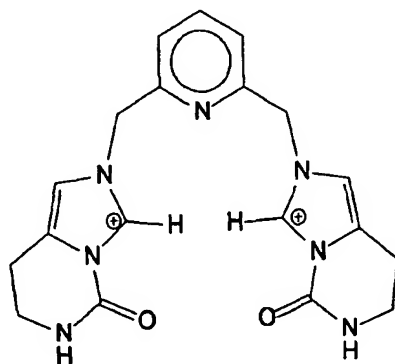
55

5. An N-heterocyclic carbene represented by the formula:



wherein Z is a heterocyclic group, and R₁ and R₂ are, independently or in combination, hydrogen or a C₁-C₁₂ organic group selected from the group consisting of alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, arylalkyl, alkylaryl, heterocyclic, alkoxy groups, and substituted derivatives thereof.

6. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, each R₁ is independently a C₁-C₆ hydroxyalkyl, and R₂ is hydrogen.
7. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, each R₁ is independently a C₂-C₃ hydroxyalkyl, and R₂ is hydrogen.
8. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, both R₁ groups together form a dimethyl phenanthroline group, and R₂ is hydrogen.
9. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, and each adjacent R₁ and R₂ together form a substituted alkyl group.
10. The N-heterocyclic carbene according to claim 9, wherein the N-heterocyclic carbene is represented by formula 26.



26

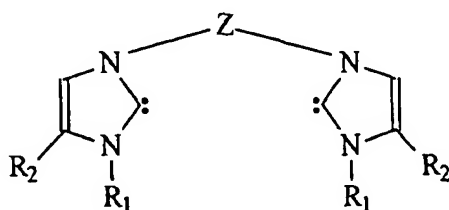
11. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, both R₁ groups form a single aryl group, and R₂ is hydrogen.
12. The N-heterocyclic carbene according to claim 11, wherein the aryl group is dimethyl phenanthroline.
13. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group and R₂ is a substituted alkyl.
14. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethylpyridine group, R₁ is a C₁-C₆ alkyl, and R₂ is a C₁-C₆ amino alkyl.
15. The N-heterocyclic carbene according to claim 5, wherein Z is a dimethyl pyrrole group, each R₁ is independently a C₁-C₆ alkyl, and R₂ is hydrogen.
16. The N-heterocyclic carbene according to claim 5, additionally complexed to silver.
17. The N-heterocyclic carbene according to claim 5, additionally complexed to a radioactive metal.
18. A method for synthesizing a radiopharmaceutical compound comprising the steps of:
 - reacting an imidazolium salt with either a transition-metal complex or a base to produce an N-heterocyclic carbene; and
 - reacting the N-heterocyclic carbene with a metal to form a metal complex.
19. A method for synthesizing an antibiotic compound comprising:
 - reacting an imidazolium salt with a transition metal complex or a base to thereby produce an N-heterocyclic carbene; and
 - reacting the N-heterocyclic carbene with a silver compound to thereby produce a silver complex with the N-heterocyclic carbene.
20. A method for treating cancer cells comprising the step of administering an effective amount of a complex of an N-heterocyclic carbene and a radioactive metal.

21. A method of creating an image of one or more tissues within a patient comprising the step of administering an effective amount of a complex of a N-heterocyclic carbene and a radioactive metal.
22. A nanofiber comprising:
 - a fiber-forming material; and
 - a metal complex of an N-heterocyclic carbene.
23. The nanofiber of claim 22, wherein the metal is Ag or a radioactive element selected from the group consisting of transition metals, lanthanide series and actinide series.
24. A method for manufacturing the nanofiber of claim 22 comprising the steps of:
 - electrospinning an electrospinnable solution that has a fiber-forming material and a metal complex of an N-heterocyclic carbene.
25. A wound dressing comprising the nanofiber of claim 22.
26. A radiopharmaceutical compound comprising a radioactive-metal complex of an N-heterocyclic carbene.
27. The radiopharmaceutical of claim 26, wherein the N-heterocyclic carbene has a peptide moiety, a polyamine moiety, or a combination thereof.
28. A method for treating a cancerous tumor comprising the step of:
 - administering an effective amount of a radioactive-metal complex of an N-heterocyclic carbene.
29. The method of claim 28, wherein the N-heterocyclic carbene has a peptide moiety, a polyamine moiety, or a combination thereof.
30. The method of claim 28, wherein the radioactive metal is an element selected from the group consisting of transition metals, the lanthanide series, and the actinide series.

31. The method of claim 28, wherein the metal is Ag, Rh, Ga, or Tc.
32. A method for synthesizing a pharmaceutical or radiopharmaceutical comprising the step of performing a carbene transfer reaction on a metal complex of an N-heterocyclic carbene.
33. The method of claim 32, wherein a silver complex of an N-heterocyclic carbene is a carbene transfer reagent.

ABSTRACT OF THE DISCLOSURE

A method for inhibiting microbial growth comprises administering an effective amount of a silver complex of a N-heterocyclic amine. A method for treating cancer cells or a method for imaging one or more tissues of a patient includes administering an effective amount of a complex of a N-heterocyclic amine and a radioactive metal. N-heterocyclic carbenes of the present invention may be represented by the formula



wherein Z is a heterocyclic group, and R₁ and R₂ are, independently or in combination, hydrogen or a C₁-C₁₂ organic group selected from the group consisting of alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, aryl, arylalkyl, alkylaryl, heterocyclic, and alkoxy groups and substituted derivatives thereof.

The following documents are a part of this application:

WO 0127365A1

CA 2386674AA

US 6110590

US 6265333

WO 0126610A1

CA 2386810AA

EP 1220694B1

WO 02100628A1

WO 0127368A1

WO 0126702A2

The attached four (4) documents are also part of this application:

Formation of Water Soluble Pincer Silver (I) Carbene Complexes:

A Novel Antimicrobial Agent.

Abdulkareem Melaiye, Richard S. Simons, Amy Milsted, Francesco Pingitore, Chrys

Wesdemiotis, Claire A. Tessier and Wiley J. Youngs

Department of Chemistry, University of Akron, Akron, Ohio, 44325

Submitted to Journal of Medicinal Chemistry



For table of content graphic

Formation of Water Soluble Pincer Silver (I) Carbene Complexes:

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Department of Chemistry, University of Akron, Akron, Ohio, 44325

Abstract

Silver (I)-2,6-bis (ethanolimidazolomethyl) pyridine hydroxide (**4a**), and silver (I)-2,6-bis (propanolimidazolomethyl) pyridine hydroxide (**4b**), are water-soluble silver (I) carbene complexes, which were synthesized in high yield by reacting silver (I) oxide with N-substituted pincer ligands **3** (**a** = 2, 6-bis (ethanolimidazoliummethyl) pyridine diiodide, **b** = 2,6-bis (propanolimidazoliummethylpyridine) pyridine dibromide). The x-ray crystal structure of **4a** is a one-dimensional linear polymer, whereas the mass spectroscopy confirms a monomer in the gas phase. A change in the anion of **4a** from a hydroxide to a hexafluorophosphate formed a silver (I) carbene complex **4c** that is dimeric in structure and insoluble in water. The bactericidal activities of the water-soluble silver (I) carbene complexes were found to be improved over that of silver nitrate.

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Formation of Water Soluble Pincer Silver (I) Carbene Complexes:

A Novel Antimicrobial Agent.

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Department of Chemistry, University of Akron, Akron, Ohio, 44325

Introduction

Silver has been recognized as an antimicrobial agent for burn wounds for decades.¹ The most widely used reagents have been silver nitrate and silver sulfonamides. In particular, silver sulfadiazine has been used in the treatment of burn wounds.² It has been assumed that the slow release of silver at the area of the superficial wound is responsible for the prevention of infection as well as aiding in the process of healing. It has also been recognized that the usefulness of silver sulfonamides has some limitations. In particular, sulfonamide-resistant organisms have been reported, limiting the clinical usefulness of silver sulfadiazine.³ Although there have been considerable advances in wound management, infections are well documented to be the leading cause of diseases in injured patients⁴. The pioneer work on metal N-heterocyclic carbenes by Ofele⁵ and Wanzlick⁶ in the 1960's and the isolation of free stable crystalline N-heterocyclic carbene by Ardunego in the early 1990's,⁷ has generated a wide interest in carbene chemistry in the last decade. Carbenes are known to be strong Lewis bases and excellent nucleophiles that bind metals better than phosphines.^{8, 9, 10} Carbenes has been proposed to bind almost all metals across the spectrum of the periodic table with better stability than phosphines.^{10, 11} There are relatively few publications on silver carbene

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complexes and their applications, aside from being used as metal transferring agents. We have reported silver (I) n-pincer type heterocyclic biscarbene complexes,^{12, 13, 14} the most recent being the synthesis of bis(silver (I) carbene) complex **2** of the pincer ligand 2,6-bis(n-butylmethylimidazoliummethyl)pyridine **1** (R = Bu)¹⁵ (Eq. 1). It was envisioned that R could be tailored to provide solubility of **2** in aqueous media and aqueous solutions of **2** would have use as anti-microbial agents.

Insert Equation 1

We report herein the synthesis of **3** (R = CH₂CH₂OH, CH₂CH₂CH₂OH) and the water-soluble silver complex **4a** and **4b**. To our knowledge, silver-carbene complexes have not been previously evaluated as anti-microbial agents. We report herein that **4a** and **4b** are useful antimicrobial agents.

Results and Discussion

Pincer ligands **3a** and **3b** are easily prepared by the reaction of 2,6-bis-(imidazolymethyl)pyridine¹⁶ with 2-iodoethanol or 3-bromopropanol respectively (Eq. 2). The IR spectra show the O-H stretching vibration at 3325 cm⁻¹. The FAB-MS spectra obtained from **3** in nitrobenzyl matrices showed [3a][I]⁺ (C₁₇H₂₃N₅O₂I) at m/z 456 and [3b][I]⁺ (C₁₉H₂₇N₅O₂Br) at m/z 436.

Pincer ligands **3a** and **3b** readily react with Ag₂O in aqueous methanol or in water to form the silver-bis(carbene) pincer complex **4a** and **4b** in high yield (Eq. 3).

Insert Equation 2.

Insert Equation 3.

The formation of **4a** and **4b** is confirmed by the loss of the imidazolium proton at 9.13 ppm for **4a** and 9.36 ppm for **4b** in the ¹H NMR spectra, and the appearance of a

resonance at 181 ppm in the ^{13}C NMR spectra. Further evidence for the formation and structure of **4a** is provided by X-ray crystallography.

Insert Figure 1

Colorless crystals of **4a** were obtained by slow evaporation of a mixture of methanol and acetonitrile solution. Interestingly, the iodide ions of **3a** are completely replaced by hydroxide ions in **4a**. In the solid state, **4a** exists as a one-dimensional linear polymer (Fig. 1). The geometry at the silver atoms is nearly linear with a C5-Ag1-C15 ($174.7(4)^\circ$) bond angle, and Ag1-C5 (2.108(11) Å), Ag1-C15 (2.060(13) Å) bond distances. Mass spectroscopy suggested that in solution and in the gas phase **4a** exists as a monomer, whereas X-ray crystallography shows that **4a** is polymeric.

Insert Figure 2

The anion exchange reaction of **4a** with aqueous ammonium hexafluorophosphate, results in the formation of **4c** (Eq. 4). In the solid state, **4c** exists as a dimer (Fig. 2). Table 1 gives a summary of the crystal data of **4a** and **4c**. The geometry of the silver atoms is nearly linear with C32-Ag1-C5 ($175.7(4)^\circ$), C22-Ag2-C17 ($174.6(3)^\circ$) bond angles and Ag1-C32 (2.070(9) Å), Ag1-C5 (2.091(9) Å), Ag2-C22 (2.064(9) Å), Ag2-C17 (2.074(8) Å) bond lengths. The nature of the anions is significant to the structural changes of **4a** versus **4c** and the choice of anion has a pronounced effect on the solubility of **4**. For example, **4a** is soluble in aqueous media whereas **4c** is not.

Insert Equation 4

Insert Table 1

The usefulness of **4a** and **4b** as antimicrobial agents was evaluated. The test organisms (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were

laboratory strains used to test a range of concentrations of the silver compounds for both the growth inhibition and minimum inhibitory concentration (MIC) determination. In both antimicrobial test methods, silver nitrate was the reference standard used, whereas the pincer ligands served as control.

A modified Kirby-Bauer method was used to obtain the sensitivity data as presented in Table 2. A constant number (volume) of bacteria were spread on the surface of a nutrient agar plate to obtain the lawn of organisms. A filter paper disc (6mm diameter) was soaked with 20 μ L solution of known concentration of the silver compounds, and placed on the lawn of organisms. The antimicrobial activity of the silver compounds was determined after an overnight incubation by measuring the diameter of the clear zone of growth inhibition of the organism around the filter disc.

Insert Table 2.

Our testing by the modified Kirby-Bauer method, confirmed **4a** and **4b** have antimicrobial properties at a level comparable to silver nitrate as shown in table 2, however the pincer ligands themselves have no activity. A limitation of the Kirby-Bauer method was observed, when approximately two fold increase in the concentration of the test silver solutions showed no significant change in the measured zone of inhibition. Lajos et al reported a similar trend for the same set of organisms when a range of silver nitrate concentration was tested.¹⁷ The diffusibility of the silver solution might have been limited by the formation of secondary silver compounds (especially silver chloride) in the test media.

The MIC is a standard microbiological technique used to evaluate the bacteriostatic activity of antimicrobial agents. In this case, the MIC was based on the total

amount of silver available and not a measure of the concentration of silver ions.¹⁸ Upon dissolving the silver complexes in the culture medium (LB broth), a precipitate of AgCl was observed in all samples. A dilution series of the supernatant portion of the silver complex solutions were prepared in LB broth, with the addition of constant volume of freshly grown organism (20 μ l) per day. The MIC was obtained by visual inspection of the turbidity of the solution as reported in Table 3. Compounds **4a** and **4b** showed better bacteriostatic activity than silver nitrate even at much lower concentration (Table 3). This can be attributed to the availability of more silver in the supernatant solutions of **4a** and **4b** than silver nitrate. Thus, **4a** and **4b** react more slowly to the chloride ions than silver nitrate in the growth medium. The contribution of the pincer ligands is significant towards reducing the formation of silver chloride in the LB broth solution compared to silver nitrate. Thus, **4a** and **4b** appear more stable than silver nitrate in the LB broth solution containing 0.1 % chloride ions, a value that is close to the physiological amount of sodium chloride (0.15M).

This is an excellent property of both **4a** and **4b** when considering silver compounds for *in vivo* application. It is important to state that although equal weights of silver compounds were used, the theoretical amount of silver ions released by **4a** and **4b** is about 2.7 times lower than that from the quantity of silver nitrate used.

Insert Table 3

When the MIC test was repeated in the presence of insoluble silver chloride, the activity of the silver compounds was enhanced, with silver nitrate performing better as shown in table 4. It has been previously reported that the presence of chloride contributes to the toxicity of silver to sensitive strains of organisms.¹⁹

Insert Table 4

The amount of silver and rate of release are known to be factors that contribute to the antimicrobial activity of silver compounds.²⁰ The MIC of **4a** and **4b** was observed to be better than silver nitrate using about the same amount of silver for each of the test compounds as shown in table 5.

Insert Table 5

The minimum lethal concentration was determined to evaluate the bacteriocidal properties of **4a** & **4b**. The clear (no growth) portion of the culture media with the lowest Ag compound concentration was used, by streaking 0.01 ml of the solution on agar plate using a sterilized loop followed by incubation at 37 °C for 24 – 48 hours. The colonies were visually counted, with the end point of the minimum bacteriocidal concentration (MBC) as no growth on the agar plate. Our test compounds showed an improved bacteriocidal effect compared to silver nitrate up to the seventh day of incubation and MBC test, with no growth observed after the tenth day of incubation and testing for the silver compounds. This is interesting considering the fact that freshly grown organisms were added per day to the culture media containing the silver compounds throughout the incubation period. The bacteriocidal and bacteriostatic properties of **4a** and **4b** are attributed to its slow decomposition of the Ag-C donor (carbene) ligand bond over time to silver metal, silver ion, AgCl and to their solubility in water. We observed a slow decomposition of **4a** and **4b** in aqueous solution after 24 hours of standing in light, and formation of silver mirror after 18 days on standing and exposure to light.

Conclusion

In conclusion, the alkanol N-functionalized silver carbene complexes **4a** and **4b** are soluble in aqueous media. The choice of anion has a pronounced effect on the solubility of **4** and its structure in the crystal forms. It has proved to be a useful antimicrobial agent and its solubility in water makes it an excellent silver compound for *in-vivo* applications. The solubility and stability of silver complexes in chloride solution are key factors that limit the use of silver complexes for *in-vivo* application. The use of Ag-C donor (carbene) compounds has demonstrated its potential as a therapeutic agent.

Acknowledgements

We thank the National Institute of Cancer, National Institute of Health (grant number 1R15 CA096739-01), The University of Akron and the Ohio Board of Regents for Financial Support. We thank the National Science Foundation (CHE-0116041) for funds used to purchase the CCD Single Crystal X-ray diffractometer used in this work. We wish to express our thanks to Dr Martha Kory of the biology Department University of Akron, for the strains of organism used.

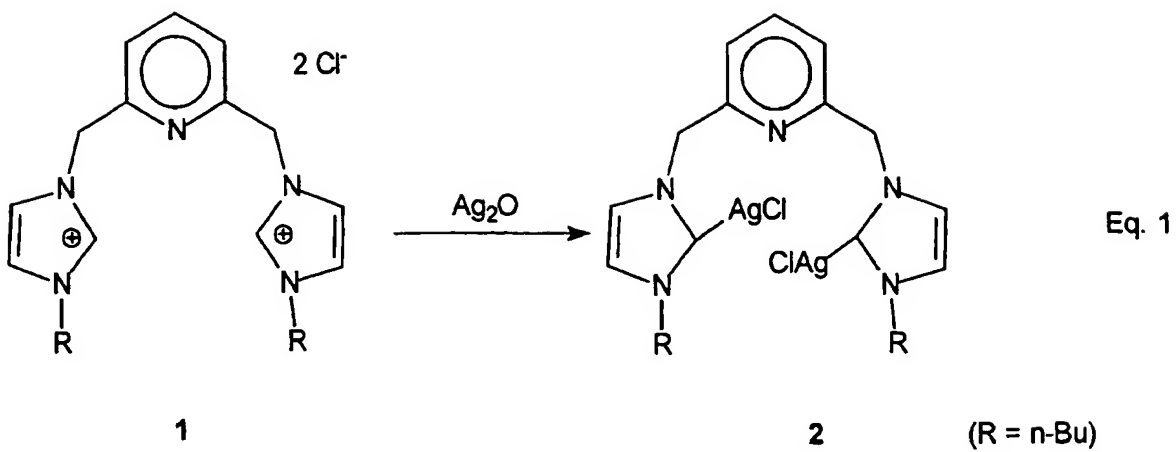
Supporting Information Available:

Complete listing of crystallography data for **4a** and **4c**, synthetic details for the new compounds **3a**, **3b**, **4a**, **4b**, **4c** and their antimicrobial testing. Spectroscopic data and experimental details are included.

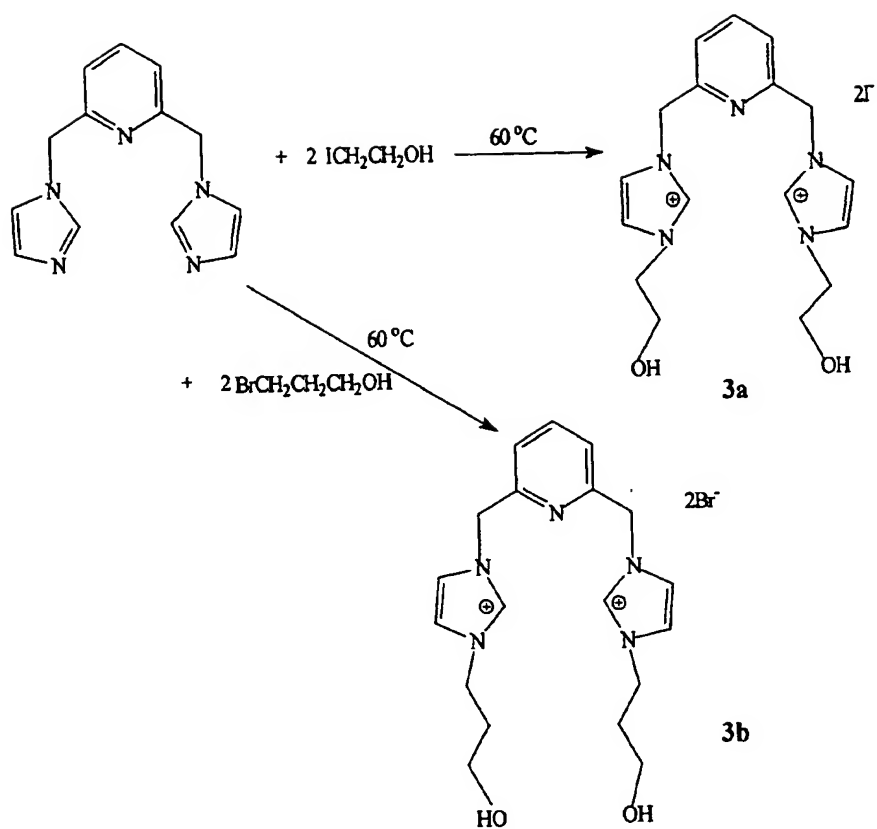
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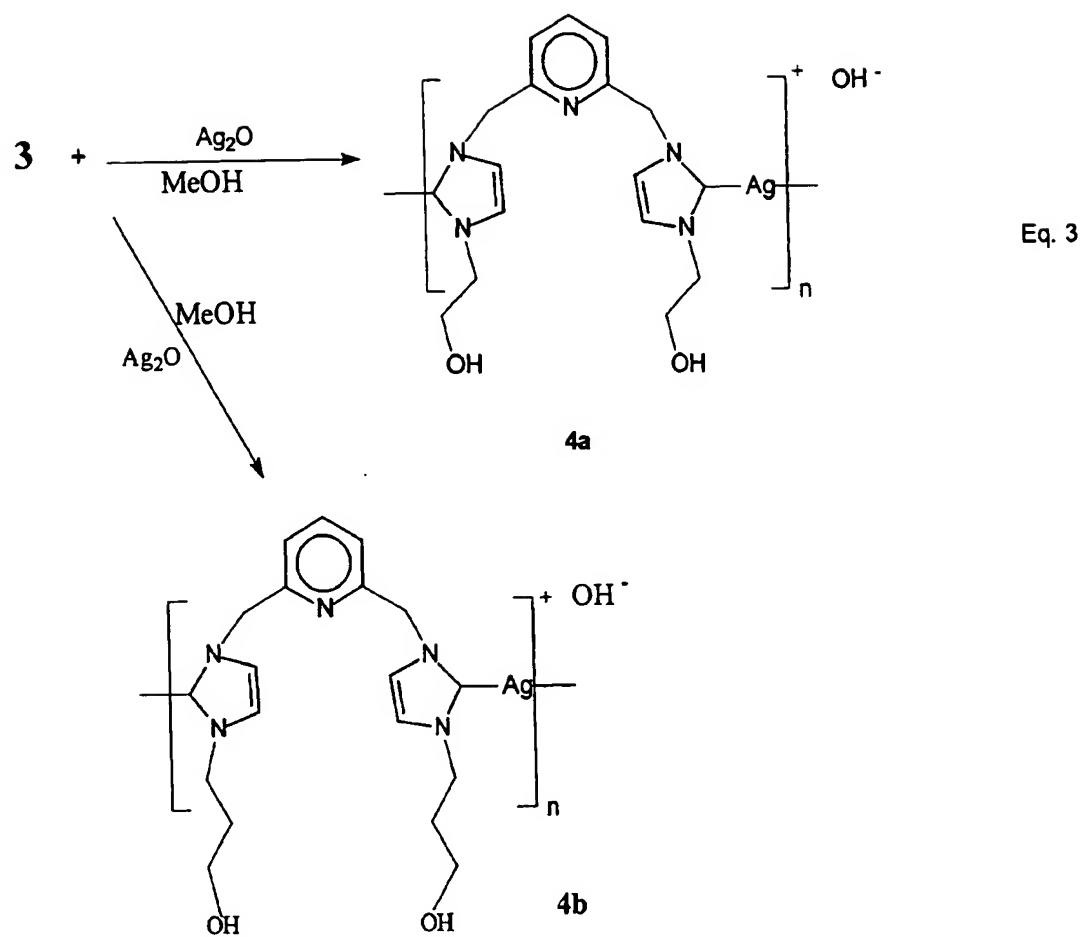


Insert For Equation 1

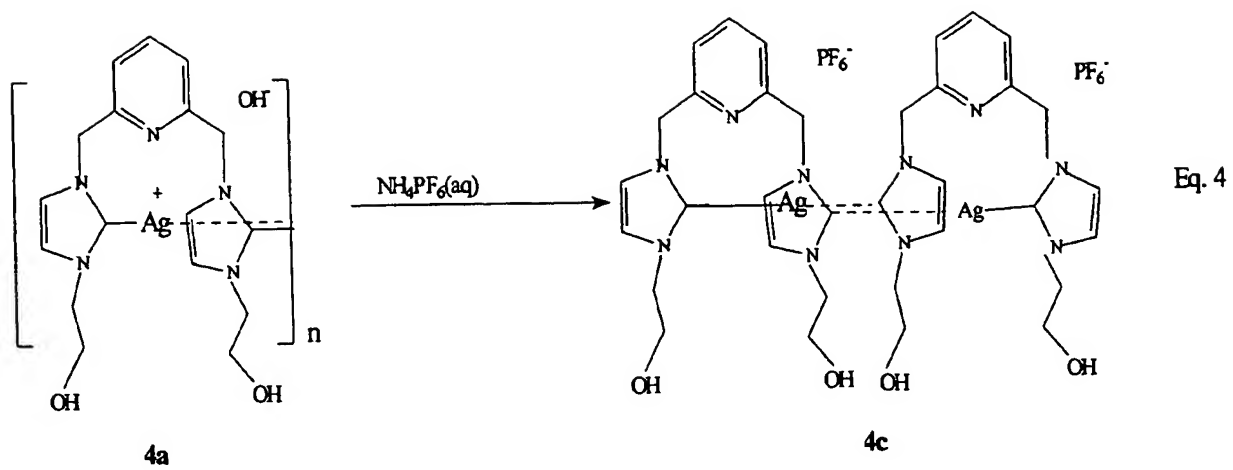


Eq. 2

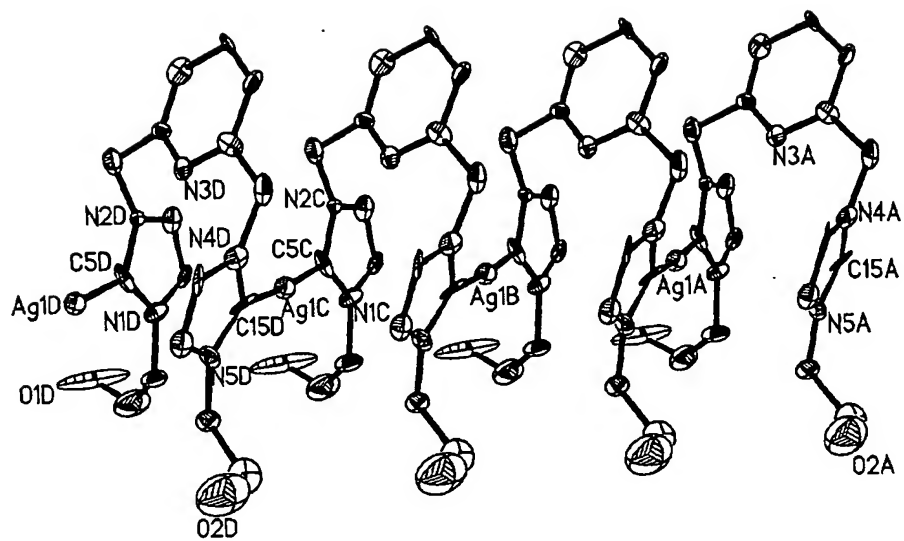
Insert Equation 2



Insert Equation 3



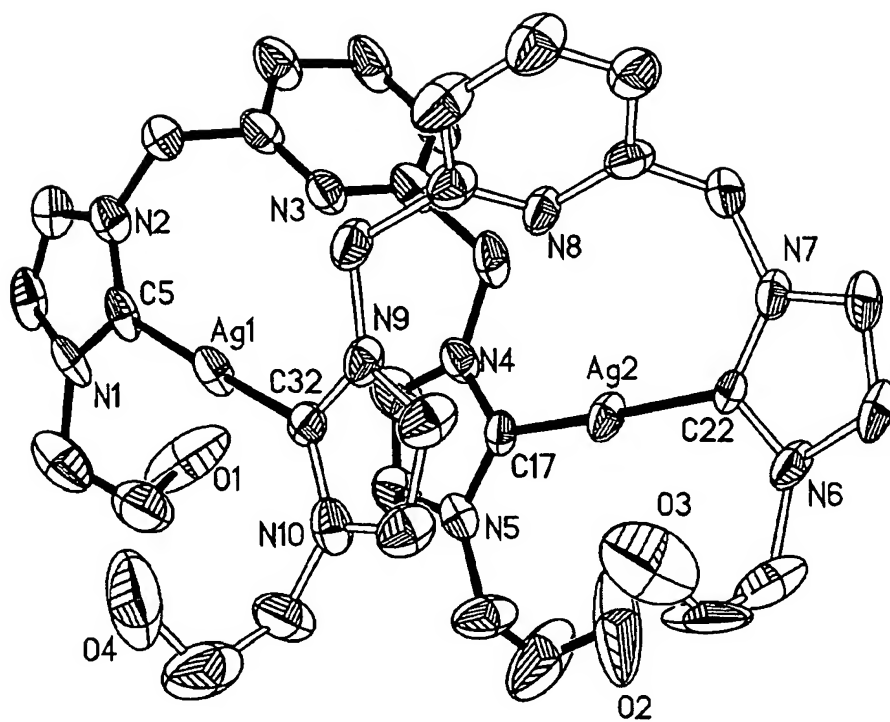
Insert for Equation 4



Insert For Figure 1

Figure 1. Thermal ellipsoid plot of **4a** with the thermal ellipsoid drawn at 50% probability level. Hydrogen atoms attached to carbon have been omitted for clarity.

Legend for Figure 1



View 1 of Figure 2



20

Figure 2. Thermal Ellipsoid plot for **4c**, with the thermal ellipsoid draw at 50% probability level. Hydrogen atoms attached to carbon are omitted for clarity

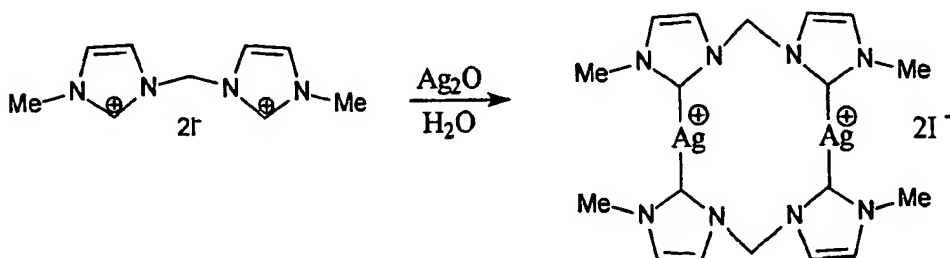
Legend for Figure 2

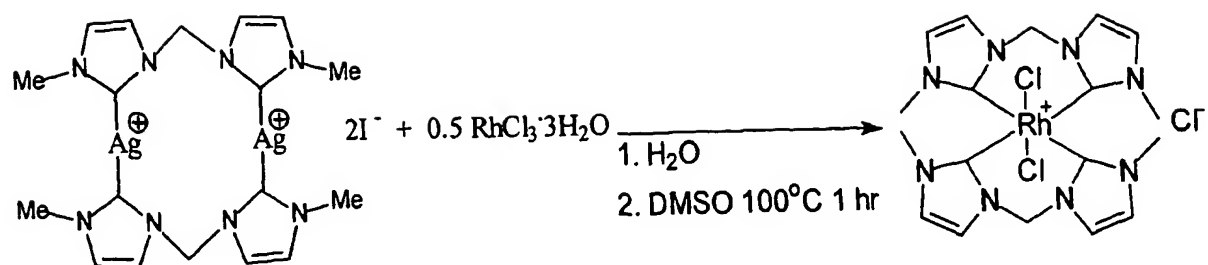
Preliminary report on the antifungal activity of the silver compounds

The anti-fungi activities of silver ethanoltrimer in reference to silver nitrate was investigated on four fungi (*Candida albican*, *Aspergillus niger*, *Mucor*, *Saccharomyce cerevisiae*) using the LB broth dilution technique. Freshly grown organism (200 μ L) was added to the broth containing dilutions of the antimicrobial agent every 24 hours after incubation at 23 °C for 7 days. The lowest concentration of the antimicrobial agent that will inhibit the visible growth of the fungi is known as the minimum inhibitory concentration (MIC) also referred to as the fungistatic activity of the antimicrobial agent. Both silver compounds contained the same amount of silver, and silver ethanoltrimer was found to have better fungistatic activities than silver nitrate on all the fungi tested. The ligand (ethanoltrimer) serves as a control for the experiment and found to have no antifungi activity. The kill effect (fungicidal activity) was also investigated by streaking each incubated broth dilutions of the silver compound on agar plate. A zero growth of the fungi on agar plate is the end point of fungicidal activity of the silver compound. Silver ethanoltrimer showed an enhanced fungicidal activity than silver nitrate by gradual release or decomposition of the silver from the ligand as shown in table 2, over the 10 days period of the experiment, compared to silver nitrate which shows a decline in its fungicidal activity with increase in the volume of organism.

The synthesis of a bis(imidazolium)Rh complex from $\text{RhCl}_3 \cdot 3\text{H}_2\text{O}$:

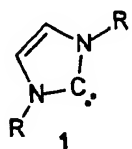
0.2150 g (0.500 mmol) of bis(1,1'-methylimidazolium)-3,3'-methylene diiodide was dissolved in 25 ml H_2O . To this solution was added 0.1390 g (0.600 mmol) of Ag_2O . Immediately a light grey precipitate formed. This suspension was stirred at RT for 15 minutes¹, and was then filtered through celite. Meanwhile 0.0329 g (0.125 mmol) of $\text{RhCl}_3 \cdot 3\text{H}_2\text{O}$ was dissolved in 10 ml H_2O . This solution was then combined with the clear, colorless filtrate from the Ag reaction, and a precipitate formed immediately. This suspension was stirred at RT for 20 minutes, and was then filtered using a M frit. After the precipitate (some combination of the ligand, AgI, and a Rh species) was dried by vacuum, it was heated in *d*-DMSO at 100°C for 1 hour. At the end, the solution was orange with a lot of solid material still present. The solution was again filtered through celite. The solvent was removed *in vacuo*, and the white and orange residue was stirred at RT in H_2O for 10 min to remove any excess ligand. Filtration of the solution yielded an orange powder. Examination of the ^1H spectrum of the orange residue in $\text{DMSO}-d_6$ revealed a mixture of the desired Rh complex and other Rh complexes in the baseline. These other Rh complexes could be the dicarbene RhCl_3 , the dicarbene RhI_3 , and/or the tetracarbene RhI_3 analogs. ^1H NMR ($\text{DMSO}-d_6$): δ 3.52 (s, 3H, CH_3), 6.71 (2d, 1H, N- CH_2), 7.45 (d, 1H, CH), 7.70 (d, 1H, CH). ^{13}C NMR ($\text{DMSO}-d_6$): δ 36.81 (CH_3), 62.18 (CH_2), 122.60 (CH), 123.56 (CH), 170.04 (d, Rh-C).





ⁱ It is crucial that this solution not stir for longer than 15 minutes or decomposition of the Ag complex, characterized initially by a brown suspension, will occur.

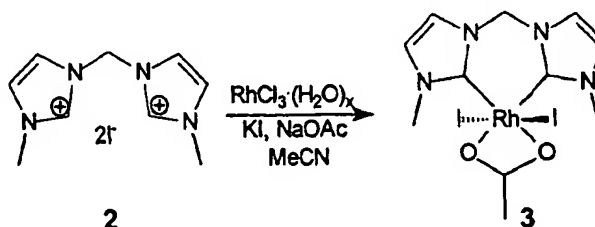
a. **Background:** The fundamental goal of this proposal is the development of new radiopharmaceuticals for the therapeutic treatment of prostate cancer with selective targeting. One of the current best therapeutic approaches to treating cancer is based on the ability of peptides to recognize receptor sites on tumor cells.¹ Currently the most promising approach for the selective targeting of cancer is the use of peptides, which are able to recognize receptor sites on tumor cells. These peptides can then be used to direct specific therapeutic (and cytotoxic) agents or imaging agents directly to the tumors minimizing exposure of non-cancerous tissue to these cytotoxic agents. Luteinizing hormone-releasing hormone LHRH has been shown to target high affinity binding sites that are present on 86% of prostate cancer cells. This would indicate that LHRH will have a high probability of success as a targeting agent for prostate cancer cells. However, LHRH high affinity binding sites are also reported in other tissues including pituitary and kidney. This suggests that cytotoxic agents targeted at prostate cancer by LHRH peptides would have the added problem of incidentally targeting pituitary and kidney tissue. It is also reported that polyamines such as spermine have been shown to selectively collect in prostate tissue. We propose the development of a dual targeted radiopharmaceutical for the selective targeting of prostate cancer cells. The new radiopharmaceutical would contain both LHRH peptides and polyamine groups to provide double targeting to prostate cancer tissue. The LHRH peptide will provide targeting to the LHRH receptors and the polyamines will provide targeting to prostate tissue. The innovative step is the double targeting of these radiopharmaceuticals. The development of a new class of radiopharmaceuticals requires that a number of critical issues be addressed. Any new radiopharmaceutical must be able to be synthesized rapidly and conveniently, preferably in a kit fashion, in physiological media in a routine clinical environment. The speed of the synthesis must be consistent with the half-life of the isotope with no further purification required and should give a product of high radiochemical purity ($\geq 90\%$). The resulting compound must have high kinetic stability in vivo.



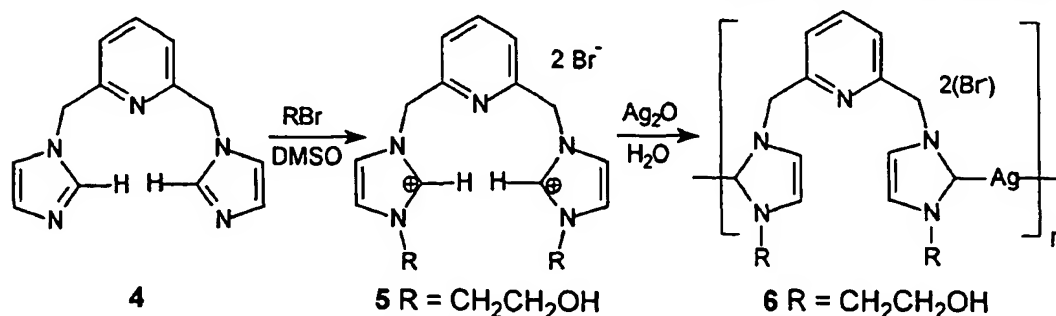
The bonding of N-heterocyclic carbenes, **1**, to transition metals is a very active area of research today,² because N-heterocyclic carbenes have been shown to bind to transition metals more strongly than do other ligands including phosphines. N-Heterocyclic carbenes have been shown to bind a variety of metals including rhodium and silver.^{2,3,4} ^{105}Rh and ^{111}Ag are beta emitters with half-lives of 1.5 days and 7.5 days, respectively. We have synthesized a variety of N-heterocyclic carbenes and are currently studying their metal complexation chemistry. **The remarkable stability of N-heterocyclic-based organometallic complexes** is their major advantage over other ligands² and this stability will be important in the applications of the metallo pincer N-heterocyclic carbenes in the field of radiopharmaceuticals.

The usefulness of complexes of radioactive metals is highly dependent on the nature of the chelating ligand.⁵ A successful metal drug must both target a specific tissue or organ as well as rapidly clear from other tissues. In addition, the target organ or tissue must have optimal exposure to the radio pharmaceutical. These needs have driven the discovery and improvement of ligand systems designed to bind radioactive metals. **Strongly chelating ligands, such as the pincer N-heterocyclic carbenes described in this proposal, can provide more advantageous radiopharmaceutical complexes.**

We have shown that the combination of **2** with $\text{RhCl}_3 \cdot (\text{H}_2\text{O})_x$ in refluxing MeCN in the presence of NaOAc and KI gives the rhodium carbene **3**. This compound has been thoroughly characterized by ^1H and ^{13}C NMR and X-ray crystallography. We have shown that the rhodium complex **3** is water stable in physiological sodium chloride solution for extended periods.

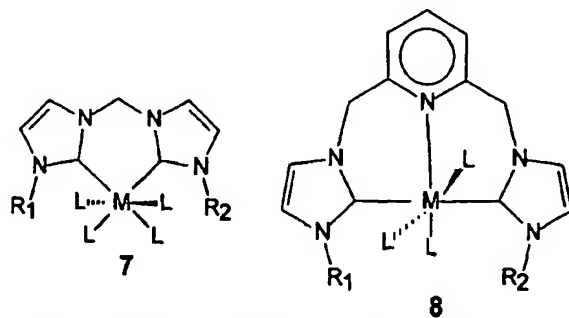


We have also developed a general synthesis for dimethylpyridine bridged pincer N-heterocyclic carbenes.⁶ For example, the combination of **4** with 2-iodoethanol or 3-bromopropanol gives **5** in high yield. The combination of the I⁻ salt of **5** with an equimolar amount of Ag₂O in water gives, after filtration, the analytically pure water soluble silver biscarbene polymer **6**. Compound **6** has been crystallographically characterized.



b. Concept: The basic concept to be explored in this proposal is that two targeting groups attached to the same radiopharmaceutical will provide better targeting than either targeting group individually. Two general types of pincer N-heterocyclic carbene chelating ligands will be examined in this project as ligands for radioactive ¹⁰⁵Rh and ¹¹¹Ag with one type represented by **3** and **7** and the second type being represented by **6** and **8**. Each of these ligand types has as their basic constituent two N-heterocyclic carbene units bridged by either methylene groups, as in **7**, or dimethylpyridine groups, as in **8**. Chelating ligands **7** and **8** will each contain two targeting groups. One of the targeting groups will be a derivative of the peptide LHRH that previously has been shown to target prostate cancer cells along with other tissues. The other targeting group will be a polyamine, such as spermine, that has been shown to target prostate tissue. The solubility and lipophilicity properties of **7** and **8** will be modified by changes in the linker groups connecting the chelating moieties and the targeting groups. Because of the strong bonding of N-heterocyclic carbenes to metals and to the chelate effect, the pincer N-heterocyclic carbene are expected to bind more strongly to the central metal than do other ligands.

c. Objectives: The ultimate aim of this research project will be to target prostate tumor cells, and make use of a chelated radioactive rhodium or silver to destroy them. The first specific aim of this proposal is the synthesis of radiopharmaceuticals, such as **7** and **8**, that have two types of targeting groups attached to the radioactive moiety. The synthesis of silver and rhodium complexes of peptide and polyamine targeted pincer N-heterocyclic carbenes will be approached by established procedures already proven to work with silver and rhodium non-targeted pincer N-heterocyclic carbene ligands. The exterior of the ligands can be functionalized by established procedures to give appropriate water solubility and lipophilicity.⁷ A variety of linker groups including PEO will allow for flexibility in positioning the chelator relative to the targeting group as well as for variation of the octanol/water partition coefficient of the compound which is relevant to the clearance through the kidneys.⁸ Based on the synthesis of **3** and **6** the synthesis of **7** and **8**, containing peptide and polyamine substituents R₁ and R₂, is reasonable.



R₁ = Linker + LHRH; R₂ = Linker + Polyamine

The second specific aim of this proposal is to investigate the efficacy of the dual targeted concept using animal studies: Study 1. Androgen-independent PC-3 human prostate cancer cells will be exposed to compounds over a range of different times and doses,

to establish conditions that will inhibit growth of the cells *in vitro*. These conditions will serve as a guide for Study 2. Study 2. Five week old CF-1 male mice (9 groups of 9 mice each) will be used to assess stability of each compound, and to determine an effective dose to be used in treating tumors *in vivo*. The silver-ligand-spermine-LHRH compound will be labeled with radioactive ^{111}Ag , and 5 μCi injected into the tail vein. The fate of the compound will be evaluated by preparing extracts of tissues and counting aliquots in a scintillation counter, available in the Biology Department. ^{111}Ag is a β emitter with a half-life of 7.47 days. All dpm values obtained will be corrected for time of isotope decay. Biodistribution will be evaluated to determine selective accumulation of compounds in the prostate gland. Each compound, at doses based on published studies and our experience with similar compounds, will be dissolved in saline and sterile filtered before intravenous (iv) injection into the tail vein. A control group of mice is injected iv with sterile saline alone. Three different doses will be used in 3 groups of mice for up to 2 weeks. Urine will be collected daily and the compound concentration determined by atomic absorption spectroscopy. NMR will be used to assess whether the chemical form of the compound has been changed. Blood samples will be collected weekly and analyzed by the same methods. Body weight will be measured daily. Results will indicate the time course of clearance of the compound from the mouse and the presence of possible metabolites of the compounds. At ages 3 days, 1 week and 2 weeks, three control and three of each experimental group will be sacrificed and the following tissues removed, weighed and collected for analysis: prostate gland, pituitary gland, pancreas, kidney, liver, heart, adrenal glands, lung, bone. After analysis for the presence of the compound, percent injected dose will be calculated for each tissue. The dose to be used in Study 3 will be based on results from the CF-1 mice. We expect to find a dose that results in preferential accumulation of compound in the prostate gland and detection of the compound there after 2 weeks. No weight loss and no premature deaths should be evident, indicating minimal toxicity to normal tissue. Study 3. Anti-tumorigenic properties of compounds will be analyzed in SCID mice with xenografted tumors from PC-3 human prostate cells. Thirty 5 week old SCID outbred mice are required. SCID mice will be housed in isolation units in a laminar flow hood, available in the Biological Resource Center at the University of Akron. Two groups of 10 mice each will be injected subcutaneously on bilateral flanks with cultured PC-3 cells (approximately 5×10^6 cells in 100 μl saline) and tumors allowed to develop for 2-3 weeks, as described.² Mice will be examined daily, and in those with tumors, the tumor dimension will be measured and tumor volume calculated. Mice will be given the dose determined previously of the silver-ligand-spermine-LHRH or the rhodium-ligand-spermine-LHRH compound by iv injection into the tail vein. After 2 weeks, mice will be weighed, terminated and tumor volumes measured. Tissues, including most of the tumors, will be collected and analyzed as in Study 2. Some tumors will be excised, weighed and fixed for routine histology, to be performed at a later date to evaluate tumor cell morphology. Results will indicate the efficacy of these new compounds as anti-prostate tumors agents.

d. **Innovation:** The major innovative concept of this proposal is the use of two different targeting groups in the same molecule of a radiopharmaceutical to enhance the targeting to prostate cancer cells. To our knowledge dual targeted radiopharmaceuticals have not been previously reported. The two different targeting groups to be used in this proposal are derivatives of the peptide LHRH and polyamines such as spermine.

e. **Relevance:** The successful outcome of this research project would mean the enhanced targeting of prostate cancer cells above what individual targeting agents can provide with a minimization of effect upon normal tissue. This would result in the ability to use increased dosage size in the treatment of prostate cancer. Advances in targeting through double targeting would also be applicable to detection and diagnosis. Enhanced targeting would be particularly important in treating metastatic cancer.

7. Abbreviations:

LHRH - Luteinizing hormone-releasing hormone The specific derivative to be investigated is Glu-His-Trp-Ser-D-Lys-Leu-Arg-Pro-Gly-NH₂ sequence. The peptide will be linked to the complex through the lysine.

Glu - Glutamate

His - Histidine

Trp - Tryptophan

Ser - Serine

Lys - Lysine

Leu - Leucine

Arg - Arginine

Pro - Proline

Gly - Glycine

NMR - Nuclear magnetic resonance spectroscopy

PEO - polyethylene oxide

CF-1 -

PC-3

SCID -

lv - intravenous

B cells -

C cells -

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